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PURIFICATION AND CHARACTERISATION OF NOVEL RECOMBINANT β-GLUCOSIDASES FROM *ASPERGILLUS* WITH APPLICATION IN BIOFUEL PRODUCTION

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PURIFICATION AND CHARACTERISATION OF NOVEL RECOMBINANT β-GLUCOSIDASES FROM *ASPERGILLUS* WITH APPLICATION IN BIOFUEL PRODUCTION

By

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Declaration

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Abstract

β-glucosidases are important components of the cellulase enzyme system in which they not only hydrolyse cellobiose to glucose, but also remove the feedback inhibition effects of cellobiose on exoglucanase and endoglucanase thereby increasing the rate of cellulose degradation to fermentable sugars. A total of 166 proteins were identified as β-glucosidases after manual BLASTp search on the *Aspergillus* comparative database from eight species. Evidence for Horizontal Gene Transfer (HGT) of bacterial origin of some β-glucosidase genes was provided by their lack of introns, absence of some fungal specific amino acid insertions in their sequences and unusual positions in phylogenetic trees showing similarities to bacterial proteins. A rapid plate assay based on Congo red methods was developed to study the optimum parameters such as pH and temperature for growth of strains and activities of the enzymes produced. Bacterial cellulose (BC) was produced by *Gluconacetobacter xylinus*. For the first time a fully detailed characterization by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), X-ray diffraction (XRD), Differential scanning calorimeter (DSC), Thermogravimetric analysis (TGA) and ^{13}Carbon Solid State Nuclear Magnetic Resonance (SSNMR) of pure BC before and after treatment with a commercially available *Aspergillus* cellulase enzyme was demonstrated. Two encoding sequences for novel *Aspergillus nidulans* hydrophobin genes ANID_05290.1 and ANID_07327 that do not fall into either the class I or class II category of hydrophobins were successfully cloned. Two encoding sequences for a novel β-glucosidase gene from an *Aspergillus niger* strain from Nigeria were amplified and cloned from genomic DNA using PCR. *Aspergillus nidulans* β-glucosidases (AN2227 and AN1804) expressed in *Pichia* were purified to homogeneity by using ammonium sulphate precipitation and DEAE-Sephadex A-50 chromatography. Both enzymes had a remarkably broad pH and temperature profile. Further experiments on the development of a technology for lignocellulose degradation based on co-production of β-glucosidase with hydrophobin for biofuel production are suggested.

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Dedication

To my parents Dn. E. A. Zakwai (Late) and Mrs Ladi E. A. Zakwai (Late)

To my wife Mercy

To the boys Jamin and Othniel

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Abbreviations

[S]	Substrate concentration
AOX1	Alcohol Oxidase 1
AOX2	Alcohol Oxidase 2
BC	Bacterial Cellulose
BGL	β-glucosidase
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool (nucleotide)
BLASTp	Basic Local Alignment Search Tool (peptide)
BMGY	Buffered Minimal Glycerol Medium
BMMY	Buffered Methanol-Complex Medium
BSA	Bovine Serum Albumin
CADRE	Central *Aspergillus* Data Repository
CAZy	Carbohydrate-Active enZyme
CBM	Carbohydrate Binding Module
CDs	Coding sequence
CFU	Colony Forming Unit
CIP	Calf Intestinal Phosphatase
cm	Centimetre
CMC	Carboxymethyl cellulose
CMCase	Carboxymethyl cellulase
CS	Cellulose synthase
CSC	Cellulose synthase complexes
ddATP	2', 3'-Dideoxyadenosine-5'-Triphosphate
ddCTP	2', 3'-Dideoxycytidine-5'-Triphosphate
ddGTP	2', 3'-Dideoxyguanosine-5'-Triphosphate
ddNTPs	Dideoxynucleotide triphosphates
ddTTP	2', 3'-Dideoxythymidine-5'-Triphosphate
DEAE	2-(Diethylamino) ethyl
DNA	Deoxy Ribonucleic Acid
DNS	3,5-Dinitrosalicyclic acid
DSC	Differential Scanning Calorimeter
EDTA	Ethylene Diamine Tetra Acetic Acid
FGSC	Fungal Genetics Stock Centre

FTIR	Fourier Transform Infrared
g	Grams
GH	Glycosyl Hydrolase
GH1	Glycosyl Hydrolase Family 1
GH3	Glycosyl Hydrolase Family 3
HGT	Horizontal Gene Transfer
HS medium	Schramm and Hestrin medium
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Daltons
Km	Michaelis constant
L	Litre
LB Broth	Luria Bertani Broth
M	Molar
MEA	Malt Extract Agar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
MSA	Multiple Sequence Alignment
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance
ORF	Open Reading Frame
PAGE	Polyacryl Amide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pDNA	Plasmid Deoxy Ribonucleic Acid
pH	Negative logarithmic of the hydrogen ion (H^+) concentration ($pH = -\log[H^+]$)
pNP	para-Nitrophenol
pNPG	para-Nitrophenyl β-D glucopyranoside
PSIPRED	Psi-blast based secondary structure prediction
RNA	Ribonucleic Acid
rpm	Revolution per minute
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SOC	Super Optimal broth with Catabolite repression
SSNMR	Solid State Nuclear Magnetic Resonance
TBE	Tris-borate-EDTA buffer
TCoffee	Tree-based Consistency Objective Function For Alignment Evaluation

TGA	Thermogravimetric Analysis
Tm	Melting temperature
TSA	Tryptone Soya Agar
UDPGlc	Uridine diphosphoglucose
URL	Uniform Resource Locator
uv	Ultra violet
Vmax	Maximum velocity or rate
w/v	Weight by volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XRD	X-ray diffraction
YASARA	Yet Another Scientific Artificial Reality Application
YEP Broth	Yeast Extract Peptone Broth
YEPD	Yeast Extract Peptone Medium
µ	Micron
µM	Micromole

Chapter 1

Introduction

1.1 Overview

With increasing demand for energy, continuous depletion of the fossil fuel energy reserves and environmental issues associated with the use of fossil fuel, attention is now focused on the development of renewable and sustainable sources of energy, particularly using cellulolytic materials. Bio-energies are energies from biomass resources and are good substitutes for fossil fuels because they are sustainable, renewable and environmentally friendly (Henry, 2010). The conversion of cellulolytic materials into fermentable sugars requires the action of endoglucanase, exoglucanase and β-glucosidase which work in concert to hydrolyse cellulose (Del Pozo *et al*., 2012; Zafar *et al*., 2011; Onyike *et al*., 2008). These enzymes belong to different glycoside hydrolase families (GH) and are classified in the Carbohydrate-Active enZyme database (CAZy, www.cazy.org) (Rytioja *et al*., 2014). Endoglucanases attack amorphous sites of the cellulose polymer, randomly yielding oligosaccharides of various lengths, while exoglucanases attack crystalline cellulose from both reducing and non-reducing ends generating glucose or cellobiose as major products (Lynd *et al*., 2002). Both endoglucanases and exoglucanases hydrolyse β-1, 4-glycosidic bonds. β-glucosidases finally hydrolyse cellobiose and other oligosaccharides into glucose. β-glucosidases are essential components of the cellulase system and are important in the complete enzymatic breakdown of cellulose to glucose. The catalysis of cellobiose is important since the accumulation of cellobiose creates feed-back inhibition. β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose (Harhangi *et al.*, 2002). Agricultural residues such as grasses, tree wastes and many other green plants whose disposal is considered as an environmental problem in Nigeria can represent large renewable resources for enzyme production by fungi (Anwar *et al*., 2014). However, the cost of obtaining sugars from lignocellulose biomass for fermentation is still high, mostly

due to low enzyme yields of producing microorganisms (Onyike *et al*., 2008). *Aspergillus niger* has been identified as one of the most efficient producers of β-glucosidase (Dan *et al*., 2000; Alriksson *et al*., 2009). Different isolates of *Aspergillus* have been reported to express β-glucosidases with characteristics properties, including differences in catalytic activity, thermostability and optimum pH (Dan *et al*., 2000; Kim[2] *et al.*, 2007). The recently completed genome projects for the genus *Aspergillus* have provided a rich resource for the identification of β-glucosidases with novel properties (Choi *et al*., 2010). Over one hundred potential candidate genes can be demonstrated in just a few species of this genus, most of which remain uncharacterised (Coutinho *et al.*, 2009).

Many different enzymes are required to achieve effective degradation of plant biomass: for example, exoglucanases, endoglucanases, β-glucosidases, endo-1,4-β-xylanases, β-xylosidases, α-l-arabinofuranosidases, acetyl xylan esterases, α-glucuronidases, pectatelyases, and endo-β-1,4-d-mannanases. Lignocellulose contains lignin, and enzymes involved in the degradation of lignin face challenges because the substrate is a large heterogeneous polymer that necessitates attack by extracellular enzymes or agents (Ruiz-Duenas and Martinez, 2009). Extracellular enzymes involved in lignin degradation are outlined in Table 1.1. Lignin does not contain hydrolysable linkages, which means that the enzymes must be oxidative (Kuhad *et al*., 2013).

Table 1.1: Enzymes involved in the degradation of lignin and their main reactions (Pandey *et al*., 2011)

Serial No.	Enzyme Activity, Abbreviation	Cofactor or Substrate, "Mediator"	Main Effect or Reaction
1	Lignin peroxidase, LiP	H_2O_2, veratryl alcohol	aromatic ring oxidized to cation radical
2	Manganese peroxidase, MnP	H_2O_2, Mn, organic acid as chelator, thiols, unsaturated lipids	Mn(II) oxidized to Mn(III); chelated Mn(III) oxidizes phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds
3	Laccase, Lacc	O_2 ; mediators, e.g., hydroxybenzotriazole or ABTS	phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators
4	Glyoxal oxidase, GLOX	glyoxal, methyl glyoxal	glyoxal oxidized to glyoxylic acid; H_2O_2 production
5	Aryl alcohol oxidase, AAO	aromatic alcohols (anisyl, veratryl alcohol)	aromatic alcohols oxidized to aldehydes; H_2O_2 production
6	Other H_2O_2 producing enzymes	many organic compounds	O_2 reduced to H_2O_2

Cellulolytic and hemi-cellulolytic multi-enzyme complexes containing some of these enzymes have been found in fungi as well as bacteria, for example, *Neocallimaxtic patriciarum* J11, *Bacillus circulans, B. megaterium* and *B. licheniformis* (Wang *et al*., 2014; Dyk *et al*., 2010; Bastioli, 2005). Interestingly, some bacteria also produce cellulose e.g. strains from the genera *Gluconacetobacter*, *Agrobacterium*, *Pseudomonas*, *Rhizobium* and *Sarcina.* However, only *Gluconoacetobacter xylinus* (*G. xylinus*) is known to produce pure bacterial cellulose by fermentation, which is identical to that produced by plants (Andrade *et al*., 2010). Bacterial cellulose is traditionally obtained in Asiatic countries by fermentation of coconut water, the main commercial product being a dessert, known as *Nata de Coco*. Bacterial cellulose is an outstanding biopolymer with unique properties such as: high water holding capacity, high crystallinity, ultrafine fibre network and high

tensile strength. This bacterial polysaccharide is secreted free of lignin, pectin, hemicelluloses and other biogenic compounds, which are associated with plant cellulose. Bacteria can therefore provide attractive alternative sources of both cellulose production and degradative enzymes (Maki *et al*., 2009).

Access to the lignocellulose complex may be facilitated by hydrophobins, which are small, surface-active non-hydrolytic proteins produced exclusively by filamentous fungi (Delmas *et al*., 2012; Littlejohn *et al.*, 2012). Hydrophobins are believed to disrupt and loosen the cellulose fibril network thereby increasing the cellulose surface area and making it more accessible to cellulase enzymes (Linder *et al*., 2005; Valdier *et al*., 2010).

The current project focuses on the purification and characterization of novel recombinant β–glucosidases from *Aspergillus* (and related species) with enhanced properties for cellulose degradation. Potential bacterial donors of cellulose degrading enzymes will also be explored for their expression in fungal hosts. Candidate genes will be cloned and expressed using simple inducible signals such as the methanol induction constructs of the yeast *Pichia*, thus allowing potentially rapid scale - up.

1.2 Literature review

1.2.1 Biomass

In the context of energy or biofuel production, biomass is essentially a biological material derived from plant based material. The concept of producing biofuels from biomass replacing the conventional petroleum fuel has recently become very important due to depletion of fossil fuel, increased environmental concerns such as air pollution and global warming (Mehdi *et al*., 2009). Many countries are now using valuable agricultural land to create biofuel crops. This is not an option in developing countries where land is needed for food cultivation, hence the interest in agricultural waste (Popp *et al*., 2014). This problem has now prompted more and more research in using cellulolytic material to generate biofuels. The by-product of fuels obtained from biomass can be in any of the three forms: solid, liquid or gas. Liquid biofuels, which are of two main types (biodiesel and bio-ethanol), are used for transportation while forestry waste and biogas produced by anaerobic digestion are used for generation of electricity and heating (Kazamia and Smith, 2014). Apart from being a renewable energy source, biofuels as an alternative to existing fossil fuel have several advantages. They are sustainable, environmentally friendly and may have a higher octane rating thereby producing lower net CO_2 emissions (Wyman, 1999).

Bioethanol is an alcohol made by fermentation, mostly from carbohydrates produced in sugar or starch crops such as corn or sugarcane. Cellulosic biomass, derived from non-food sources such as trees and grasses, can also be used for ethanol production. Cellulose, the most abundant biomass on earth, is a long chain of D-glucose with β- 1, 4 linkages and forms a linear polymeric chain of 10,000 glucose residues (Shawn and Roger, 2003). Cellulose is not in a form suited for direct human needs (i.e. for food or fuel), unless it is modified. This is because the β- 1,4 linkages of cellulose are not hydrolysed by α-amylases which in humans hydrolyse α- 1,4 linkages (Lehninger *et al.*, 1993). The production of ethanol from lignocellulosic biomass involves pre-treatment, hydrolysis (saccharification),

fermentation and ethanol recovery through distillation and evaporation (Figure 1.1) (Van Zessen *et al*., 2003; Mehdi *et al*., 2009). Thus, proper pre-treatments of biomass material could change them from liabilities into assets.

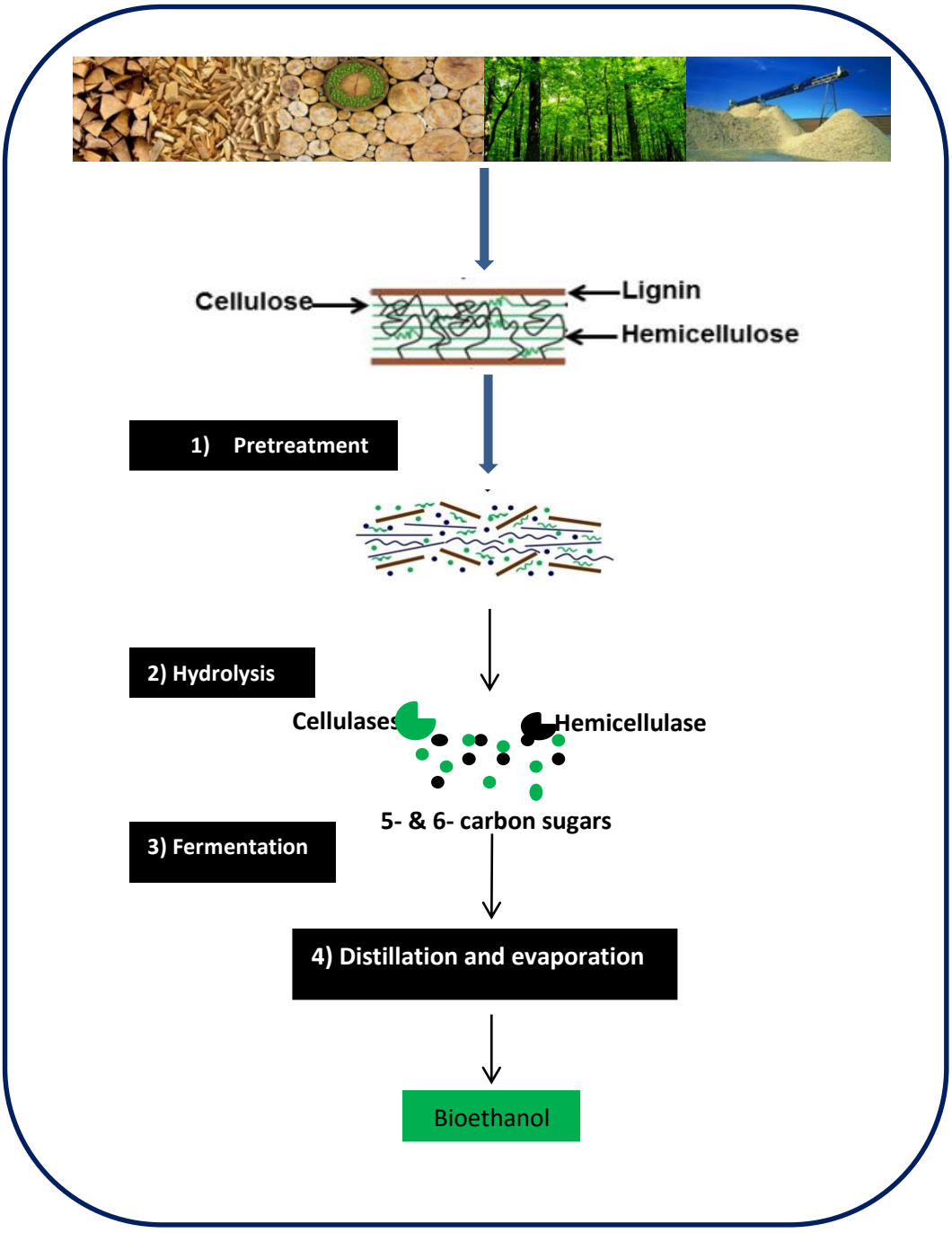


Figure 1.1: Schematic picture for the conversion of lignocellulosic biomass to ethanol (Mehdi *et al*., 2009).

1.2.2 Pre-treatment

Pre-treatment helps the separation of biomass into lignin, hemicellulose and cellulose and is necessary because cellulose, which is the required substrate, is often buried and locked within lignin and hemicellulose framework. The composition of the lignocellulosic material can vary greatly among various sources (Table 1.2).

Table 1.2: Composition of some common sources of lignocellulosic materials (Sun and Cheng, 2002)

Source	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Corn cobs	15	35	45
Wheat straw	15	50	30
Grasses	10 – 30	35 – 50	25 – 40
Waste papers from chemical pulps	5 – 10	10 – 20	60 – 70
Paper	0 – 15	0	85 – 99
Newspaper	18 – 30	25 – 40	40 – 55
Leaves	0	80 -85	15 – 20
Nut shells	30 – 40	25 – 30	25 – 30
Cotton seed hairs	0	5 – 20	80 – 95
Hardwood stems	18 – 25	24 – 40	40 – 55
Softwood stems	25 – 35	25 – 35	45 – 50
Coastal Bermuda grass	6.4	35.7	25
Switch grass	12	31.4	45
Primary wastewater solids	24 – 29	NA	8 – 15
Swine waste	NA	28	6
Solid cattle manure	2.7 – 5.7	1.4 – 3.3	1.6 – 4.7
Sorted refuse	20	20	60

NA: Not applicable.

The aim of pre-treatment is to maximize sugar yield by removing the lignin component, separate hemicellulose from cellulose, decrease the crystallinity of cellulose and increase the pore size of the cellulose to facilitate the penetration of hydrolytic enzymes (Figure 1.1) while minimizing chemical destruction of fermentable sugar (Mani *et al.*, 2002). Pre-treatment is also important in order to make the cellulose physically available for breakdown, by expanding the polysaccharide matrix (De-Vries and Visser, 2001). Although, the available technologies used for lignocellulosic pretreatment facilitate the breakdown process, there is still a bottleneck affecting an efficient scale-up of the technology. This bottleneck includes high costs of production, low specific activity of the enzymatic cocktails and the limited number of companies producing the enzymes (Del Pozo *et al*., 2012).

The different types of pre-treatment methods include: physical processes, physico-chemical processes, chemical processes and biological processes. Alkaline hydrolysis, acid hydrolysis, oxidative delignification and ozonolysis are currently the common chemical methods used for the pre-treatment of biomass. Alkaline and acid pre-treatment methods are the most frequently used (Mani *et al*., 2002).

1.2.2.1 Alkaline pretreatment

Ammonia (NH_3) and sodium hydroxide (NaOH) are often used as alkaline solutions for the pretreatment of biomass materials. Alkaline pretreatment methods can be effectively utilized for agricultural crop residues such as sawdust, sugar cane bagasse, corn cobs, corn straws, leaves etc. (Bjerre *et al*., 1996). Iyer *et al*., (1996) used the alkaline pretreatment method (NH_3) for treating corn cobs. They treated the sample with 2.5 – 20% ammonia solution at 170^{o}C for one hour and were able to achieve 60 – 80% lignin removal for corn cobs. Abu *et al.,* (2001) and Namita *et al.,* (2012) observed that the highest amount of cellulose and cellulases were produced in biomass pre-treated using the alkaline pre-treatment method and had comparable production of cellulose to that in

Carboxymethylcellulose (CMC) medium. In another fractionation of corn cobs using alkaline pretreatment reported by Cao *et al*., (1996), they reported that the majority of lignin was solubilized and separated from cellulose and hemicellulose. After the separation of hemicellulose from the cellulose fraction, cellulose was used as a substrate in the process of ethanol production using a thermo-tolerant yeast strain as biocatalyst.

When lignocellulolytic material is treated with dilute NaOH, the internal surface area of the material increases by swelling and causes a decrease in degree of polymerization, separation of structural linkages between carbohydrate and the disruption of lignin structure (Fan *et al*., 1987). The alkaline pre-treatment hydrolysis mechanism is believed to be saponification of intermolecular ester bonds cross-linking lignin and hemicelluloses (Tarkow and Feist, 1969).

1.2.2.2 Acid hydrolysis

In lignocellulosic acid hydrolysis, different types of acids (diluted or concentrated) can be used. The most common acids used are: sulphuric acid (H_2SO_4), hydrochloric acid (HCl), phosphoric acid (H_3PO_4), formic acid (CH_2O_2), nitric acid (HNO_3) and hydrofluoric acid (HF) (Lenihan *et al*., 2010; Galbe and Zacchi, 2002). The concentration of the acid in the hydrolysis process is usually within the range of 10 – 30% and the process is carried out at low temperatures producing high yields of cellulose (Iranmahboob *et al*., 2002). One of the major advantages of acid hydrolysis is that the acid used penetrates lignin, breaking down hemicellulose and cellulose to fermentable sugars without any preliminary pretreatment of biomass. The main problem of biomass acid hydrolysis is that it causes corrosion problems to the equipment used, and in the case where dilute acid hydrolysis is carried out, a high temperature is required to achieve appreciable amounts of cellulose conversion (Balat *et al*., 2008).

1.2.2.3 Enzymatic hydrolysis

The use of enzymatic methods in the breakdown of cellulose into fermentable sugars has advantages compared to using just chemical methods. This is simply because of the fact that enzymes are highly specific in their action and they work under milder conditions. Though quite a number of microorganisms are able to produce cellulose degrading enzymes, very few are capable of producing a large amount of cell free enzymes that will completely hydrolyse cellulose (Shawn and Roger, 2003). Cellulose degrading microorganisms include aerobic bacteria and fungi which secrete soluble extracellular enzymes, known as the non-complex cellulase system, while anaerobic cellulolytic bacteria produce complex cellulase systems, called cellulosomes and grow optimally at or below 60^{o}C (Blumer-Schuette *et al*., 2014; Dixon, 2013; Sun and Cheng, 2002). A fungus, *Trichoderma reesei* (*T. reesei*) is an efficient producer of extracellular enzymes and is one of the non-complex cellulase systems that have been fully investigated (Bonner, 1979; Bayer *et al*., 1998; Nakari-Setala *et al*., 2009; Jun *et al*., 2011; Saranraj *et al*., 2012; Tiwari *et al*., 2013). *T. reesei* produces seven endoglucanases, two exoglucanases and a number of β-glucosidases. Unfortunately the amount of β-glucosidases naturally produced by *T. reesei* is very low and is not efficient in the hydrolysis of cellulose into fermentable sugars (Yang *et al*., 2011). Nevertheless, there is an extensive documentation about the conversion of cellulosic waste to sugar by cellulases from selected strains of another species in the same genus, *T. viride* (e.g. Mandel, 1974; Karen *et al*., 1996).

In fungi, independent "extracellular cellulases" act in concert to hydrolyse cellulose whereas in bacteria "cellulosomes" made up of non-enzymatic proteins associated with enzymatic subunits act together to degrade lignocellulose (Doi *et al*., 2003). Cellulosome "overhangs" are produced on the cell wall of the cellulolytic bacteria grown on cellulosic materials. These "overhangs" are stable enzyme complexes tightly bound to the bacterial cell wall but flexible enough to bind strongly to cellulose. Bacteria produce multiple

enzymes which self-assemble into complex cellulosomes to degrade plant cell walls to access the plant's nutritious sugars (Dixon, 2013). All the hydrolytic proteins found on the cellulosome are held together by non-catalytic scaffolding proteins which are a functional unit of cellulosomes and contain multiple copies of cohesins that interact selectively with domains of the enzymatic subunits and carbohydrate binding modules (Blumer-Schuette *et al*., 2014). These have complementary cohesins, called dockerins, which are specific for each bacterial species (Mechaly *et al*., 2001; Lynd *et al*., 2002; Dixon, 2013).

1.2.3 Structure of cellulose

D-glucose is the building block of cellulose and exists mostly in the more stable hemiacetal form, which has two isomers: α- and β-glucose. Cellulose molecules are unbranched chains formed by β-glucose and are insoluble. The cellulose polysaccharide consists of a linear chain of several hundred to over ten thousand β-(1 – 4) linked D-glucose units (Figure 1.2) and it is the most abundant natural polymer on earth (Del Pozo *et al*., 2012; Yoshihiko *et al*., 2005). Cellulose is the primary structural component of the plant cell wall and some species of bacteria also secrete cellulose (Andrade *et al*., 2010). The polymeric molecules of cellulose are arranged in micro-fibrils to give strength to plant cell walls (Coutinho *et al*., 2009). The cellulose chain is arranged in such a way that the reducing end is at one end and non-reducing end at the other end. These sub-structures result in micro-fibrils that contain cellulose chains ordered in highly crystalline and amorphous regions (Attala *et al*., 1984). The amorphous region is easily hydrolysed while the crystalline region is not (Muhlenthaler, 1967).

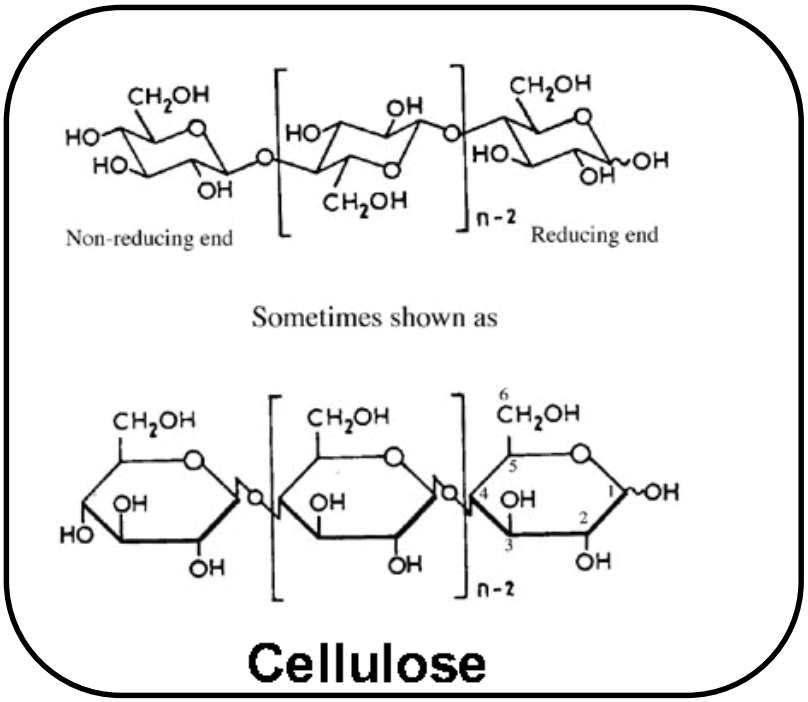


Figure 1.2: Structure of cellulose (fibersource.com)

Cellulose is usually associated with other polysaccharides such as lignin and hemicellulose and is usually buried and locked by their networks. Lignin and hemicellulose support the plant by reducing the availability of cellulose for enzymatic penetration and activity (Jacobus, 2001).

1.2.4 Hydrolysis of cellulose

Cellulose is hydrolysed by a group of enzymes known as cellulases. Cellulases are made up of three basic enzymes which are endoglucanase, exoglucanase and β-glucosidase (Han *et al.*, 1995; De-Vries and Visser, 2001) which work cooperatively in the hydrolysis of cellulose (Del Pozo *et al.*, 2012; Onyike *et al*., 2008). Endoglucanases attack amorphous sites of the cellulose, randomly yielding oligosaccharides of various lengths, while exoglucanases attack crystalline cellulose from both reducing and non-reducing ends (Figure 1.3) generating glucose or cellobiose as major products (Lynd *et al*., 2002; Onyke

et al., 2008). Glycosyl hydrolases are classified on the basis of substrate specificity, mechanism of action (retention or inversion), mode of action (endo or exo) and amino acid sequence similarities. Even by 1997, 60 sequence-based Glycosyl hydrolases were known and the classification has been frequently updated (Henrissat and Davies, 1997). GH61 (Figure 1.3) are accessory enzymes that are co-expressed with endoglucanase, exoglucanase and β-glucosidase for efficient hydrolysis of cellulose during growth on cellulosic substrates (Amore *et al*., 2013); (full description is given in section 1.2.7). Both endoglucanases and exoglucanases hydrolyse β-1, 4-glycosidic bonds. β-glucosidases hydrolyse cellobiose and other oligosaccharides into glucose. β-glucosidases are essential components of the cellulase system and are important in the complete enzymatic breakdown of cellulose to glucose. Although the three cellulases differ in the site of attachment, the mechanism used to remove the sugars from the cellulose chain remains identical (Lynd *et al.*, 2002).

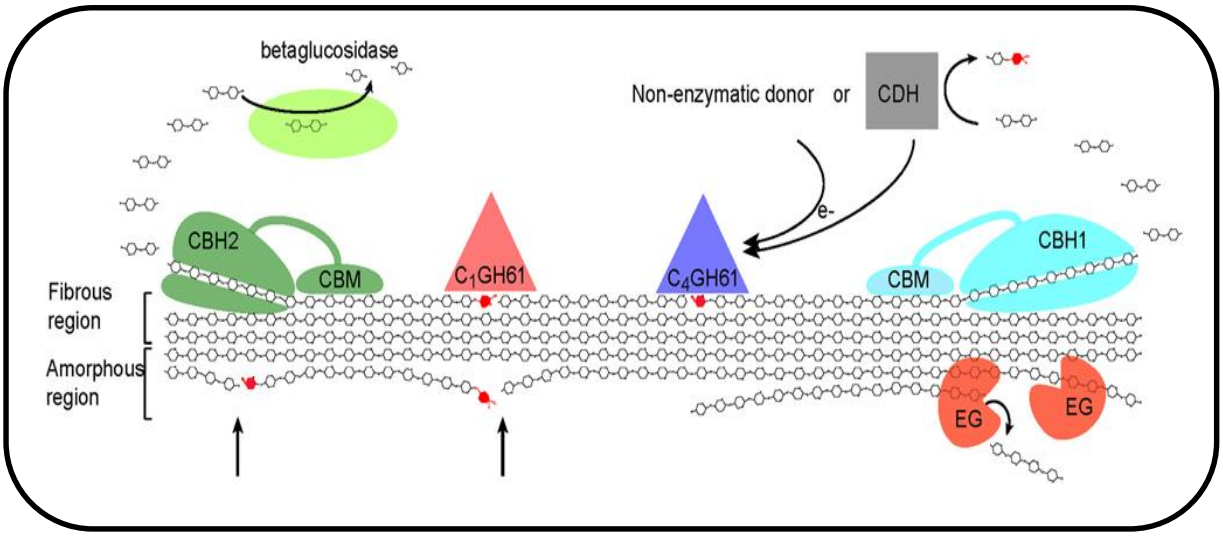


Figure 1.3: Current view (Horn *et al*., 2012) on fungal enzymatic degradation of cellulose. Abbreviations: EG, endoglucanase; CBH, cellobiohydrolase; CDH, cellobiose-dehydrogenase; CBM, carbohydrate-binding module. The figure also shows a C1 and a C4 oxidizing GH61 which would generate optimal (i.e. non-oxidized) ends for the CBH2 and the CBH1, respectively (oxidized sugars are coloured red). Note that the combined action of C1 and C4 oxidizing enzymes may produce native cello-oligosaccharides from the middle of the cellulose chain.

It is quite expensive to produce biofuel from cellulose rather than from starch. Therefore, hydrolysis of cellulose becomes the bottleneck for bringing down the cost of biofuel production from cellulolytic material (Sørensen *et al*., 2013).

1.2.5 Microorganisms involved in cellulose degradation

Microorganisms, plant and insect sources have been widely exploited for the production of cellulases. Although a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell free enzymes capable of completely hydrolysing cellulose (Shawn and Roger, 2003). For instance, microorganisms associated with cellulose hydrolysis include bacteria such as *Bacillus spp*., *Clostridium spp*. (Menendez *et al.*, 2015), *Actinomycetes* spp, *Thermoactinomyces spp.* and *Streptomyces spp*. (Irwin *et al.*, 2000), fungi such as *Trichoderma spp*. and *Aspergillus spp*. (Shawn and Roger, 2003; Murashima *et al.*, 2002; Zhang *et al.*, 2009). The main obstacle

that opposes cellulose enzymes is their restrained access to cellulose that is locked and buried within lignocellulose micro-fibrils. It has been proposed that these unreachable regions are agitated or unlocked by non-hydrolytic proteins which disrupt the packaging of the cellulose fibril network (Arantes and Saddler, 2010). The cellulose hydrolysis process of such proteins is said to interact together with cellulase enzymes to hydrolyse cellulose (Figure 1.3) (Horn *et al*., 2012).

1.2.6 Hydrophobins

Hydrophobins are a cysteine rich low molecular mass (≤20 kDa) group of proteins approximately 50 to 125 amino acids in length (though they can be over 400 amino acids in length when including poly-hydrophobins) that are expressed only by filamentous fungi and are secreted into liquid media and also are present at the surface of aerial mycelia (Bayry *et al.*, 2012; Wessels, 1997; Littlejohn *et al.*, 2012). They are characterized by moderate to high levels of hydrophobicity and the presence of eight conserved cysteine (Cys) residues (Bayry *et al.*, 2012).

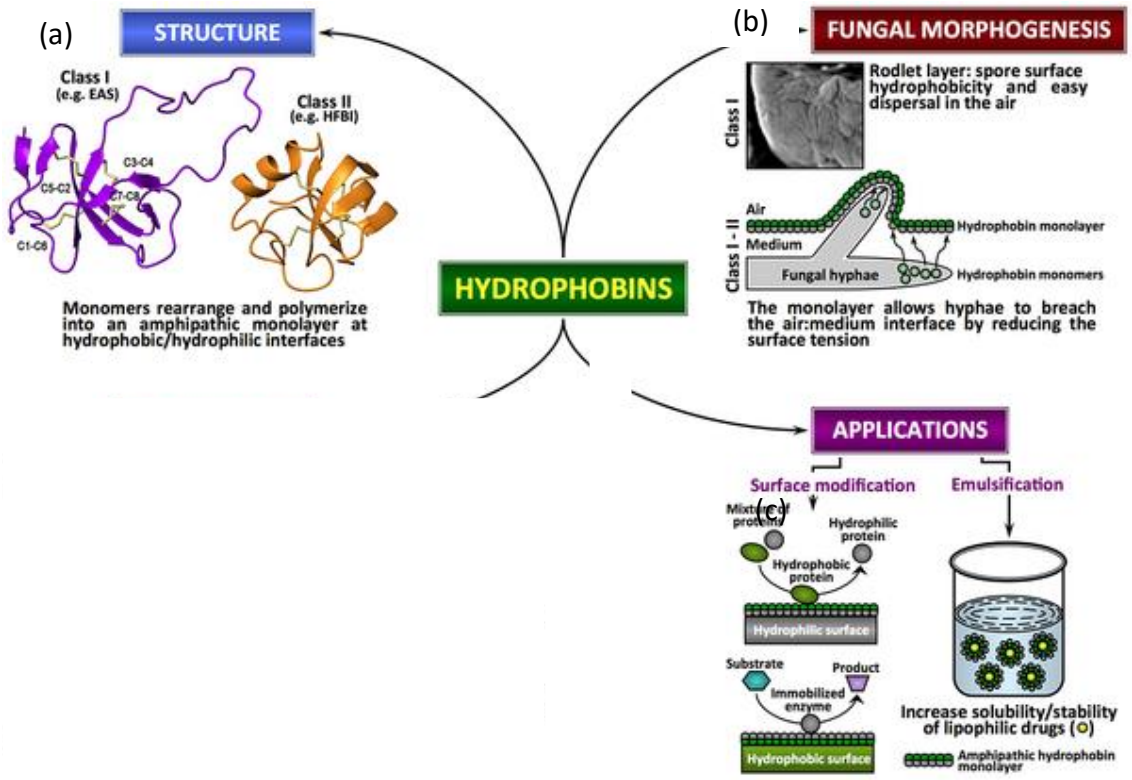


Figure 1.4: Fungal Hydrophobins (Bayry *et al*., 2012).

Hydrophobins were traditionally divided into two classes based on hydropathy plots, solubility and the type of layer they form after aligning the cysteine residues in their sequences. In class I, hydrophobins have a significantly different amino acid arrangement from class II hydrophobins (Figure 1.4a) (Wessels, 1997) and considerable discrepancy is seen in the inter-Cys spacing. The class I hydrophobins are gathered into highly insoluble polymeric monolayers made of fibrillar structures called rodlets, which are stable and can only be dissolved by harsh acid treatments and under appropriate conditions, the soluble form can polymerize back to rodlets (Bayry, *et al*., 2012; Wösten, 2001). In class II hydrophobins, the monolayers formed lack the fibrillar rodlet morphology primary sequence and the inter-Cys spacings are more conserved and can be solubilized by

detergents and solvents (Bayry, *et al.*, 2012; Wösten, 2001). Figure 1.4 summarizes the key characteristics of hydrophobins. More recently bioinformatics techniques have identified hydrophobins intermediate in structure which fall between class I and class II (Littlejohn *et al*., 2012).

Hydrophobins have many potential biotechnological applications in the pharmaceutical and nanotechnology industry, for example as biosurfactants (Scholtmeijer *et al*., 2001), protein immobilizers and separators (Joensuu *et al*., 2010; Janssen *et al*., 2004), cosmetics improvement (Vic, 2003) and as foam stabilizers (Murray, 2007; Green *et al*., 2013). Studies by Delmas *et al*., (2012) reported that genes encoding hydrophobins were greatly induced when the carbon source for *Aspergillus* was changed from glucose to lignocellulose thus suggesting that hydrophobins may have a role together with other lignocellulolitic enzymes in the degradation of lignocellulose.

Most of the cellulose hydrolysing enzymes from fungi are said to be modular proteins with both catalytic and carbohydrate binding modules (CBM). The CBM, which are non-catalytic domains, are thought to be involved in enzyme concentration on the surface of the substrate, substrate targeting and disruption of non-hydrolytic crystalline substrate (Lambertz *et al*., 2014; Valdeir and Jack, 2010; Arantes and Saddler, 2010). Additionally it has also been proposed that regions of cellulose that are inaccessible are usually loosened or disrupted by non-hydrolytic proteins, making them more accessible to cellulase enzyme complexes by increasing their surface areas (Valdeir and Jack, 2010).

Mandels and Reese (1964) introduced the $C_1 - C_x$ model and hypothesized that C_1 (which is the unknown component of the cellulase system) opens up the cellulose matrix allowing the accessibility of the hydrolytic enzymes (C_x) to hydrolyse cellulose (Arantes and Saddler, 2010). Hydrophobins could play this important role of C_1. The self-assembling property of hydrophobins at hydrophilic-hydrophobic interfaces enables the formation of amphipathic membranes which change the surface hydrophilic or hydrophobic nature of

their target surfaces (Wang *et al*., 2005), and by so doing, hydrophobins help to produce aerial structures (Talbot, 1999). Attachment of hydrophobins to lignocellulose may induce surface modifications thereby hydrolytically loosening or disrupting the fibril network.

1.2.7 Cellulases

Cellulases are a group of enzymes that are produced by fungi and bacteria that catalyse the hydrolysis of cellulose and other related polysaccharides to yield oligosaccharides of various lengths and monosaccharides (simple sugars) such as β-glucose. Fungi produce three basic types of extracellular enzymes that are involved in the hydrolysis of cellulose: endoglucanase (EC 3.2.1.4) which is also referred to as carboxymethyl cellulase (CMCase), exoglucanase (EC 3.2.1.91) also known as cellobiohydrolase and β-glucosidase (EC 3.2.1.21) also known as cellobiase. The conversion of cellulolytic materials into simple fermentable sugars requires the action of these three cellulase enzymes which work together to hydrolyse cellulose, yielding fermentable sugars (Onyike *et al.,* 2008). Endoglucanases are generally monomers with none or low glycosylation and usually attack amorphous sites of the cellulose randomly yielding oligosaccharides of various lengths. Exoglucanases are also monomers with none or low glycosylation and attack crystalline cellulose from both reducing and non-reducing ends generating glucose or cellobiose as major products (Lynd *et al*., 2002). Both endoglucanases and exoglucanases hydrolyse β-1, 4-glycosidic bonds. β-glucosidases hydrolyse cellobiose and other oligosaccharides into glucose. Some β-glucosidases have a simple monomeric structure; some others are dimeric or even trimeric and most are glycosylated (Mehdi *et al*., 2009). β-glucosidases are essential components of the cellulase system and are important in the complete enzymatic breakdown of cellulose to glucose. The catalysis of cellobiose is important since the accumulation of cellobiose creates a feedback inhibition (Mallerman *et al*., 2015). β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and

is a rate limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose (Harhangi *et al*., 2002).

1.2.8 β-glucosidases

β-glucosidases (also known as cellobiase, β-D-glucosidase, β-glucoside glucohydrolase, p-nitrophenyl β-glucosidase, and β-1,6-glucosidase; EC 3.2.1.21) catalyze the hydrolysis of terminal non-reducing residues in β-D-glucosides by the transfer of a glycosyl group between oxygen nucleophiles to release glucose (Cox *et al*., 2000). β-glucosidases are well distributed in nature and they play important roles in a large number of biological processes (Mallerman *et al*., 2015). In humans for instance, β-glucosidase is involved in the hydrolysis of glucosyl ceramides (Lieberman *et al*., 2007). In plants, β-glucosidase is involved in the synthesis of β-glucan during cell development, defense mechanisms, fruit ripening and pigment metabolism while in microorganisms it is involved in cellulase induction and cellulose hydrolysis (Brozobohaty *et al*., 1993; Tomme *et al*., 1995; Bisaria and Mishra, 1989).

1.2.8.1 Classification of β-glucosidases

There is no single well defined method for the classification of β-glucosidase, and four methods for their classification appear in the literature. β-glucosidases are classified on the basis of substrate specificity, encoding gene nucleotide sequence identity, mechanism of action and localization within the cell (Sorensen *et al*., 2013; Singhania *et al*., 2013; Henrissat and Bairoch, 1996; CAZypedia).

Based on substrate specificity, β-glucosidases have conventionally been divided into cellobiase (with specificity for cellobiose), aryl-β-glucosidase (with specificity for p-nitrophenyl-β-D-glucopyranoside, pNPG) and broad specificity β-glucosidases (Sorensen *et al*., 2013). CAZy classification of Glycosyl hydrolases (GH) has placed β-glucosidase in family GH1 and GH3 based on their amino acid sequences but they can also be found in GH family 5, 9, 17, 30 and 31 (Singhania *et al*., 2013; CAZypedia; Cantarel *et al*., 2009;

Opassiri *et al*., 2007; Henrissat, 1991). GH 1 family β-glucosidases include β-glucosidases of mammalian, bacterial and plant origin which in addition to β-glucosidase activity also have galactosidase activity, while GH 3 family β-glucosidases include β-glucosidases from fungi, bacteria and plants.

Based on mechanism of action, hydrolysis of β-1,4-glycosidic bonds by β-glucosidase is carried out by the overall retaining double displacement mechanism. Two catalytic carboxylic acid residues at the active site facilitate the reaction with one carboxylic acid acting as a nucleophile and the other as an acid/base catalyst (Sørensen *et al*., 2013; Mehdi *et al*., 2009). Under physiological conditions, β-glucosidase catalyses the transfer of glycosyl groups between oxygen nucleophiles resulting in the hydrolysis of β-glucosidic bonds between carbohydrates residues while under defined conditions, synthesis between different molecules can occur by reverse hydrolysis and transglycosylation (Singhania *et al*., 2013).

Although the functional prediction of CAZy is difficult because of low sequence identity, Busk and Lange (2013) reported new alignment independent techniques to recognize genes for GH1 and GH3 families and to also identify related sequences in highly different proteins. However, the Henrissat and Bairoch (1996) scheme of classification by nucleotide identity is the most accepted method of β-glucosidase classification. The sequence-based classification is very important and useful in the characterization of β-glucosidase from a structural viewpoint (Lima *et al*., 2013).

In order to increase the efficiency of cellulose hydrolysis, it is important to prepare a cocktail of cellulose hydrolysis enzymes supplemented with β-glucosidase. One strategy could involve the over-expression of β-glucosidase genes for example, using the *Pichia pastoris* (*P. pastoris*) methanol-induced promoter to drive its expression (Liu *et al*., 2013). Genes for glucose tolerant β-glucosidase can be isolated and cloned into a suitable vector and then engineered for expression.

1.2.8.2 Microbial production of β-glucosidase

Although cellulases are distributed throughout the biosphere, they are more commonly found in fungi and bacterial sources, although only a few of these microorganisms produce significant quantities of β-glucosidase. The production of microbial β-glucosidase can be achieved by the submerged fermentation (SmF) technique and/or solid state fermentation (SSF). Submerged fermentation (SmF) has been employed for commercial production of microbial enzymes because of some advantages such as better sterility, ease of process monitoring and automation. Solid state fermentation (SSF) is also popular in the case of fungal fermentations for high volume/low value enzymes because it has better productivity, cheaper crude substrate utilization, low energy requirement, lack of foam build up and higher product concentration. (Kovacs *et al*., 2008).

For example, *Aspergillus oryzae* (*A. oryzae*), *A. niger* and *Phanerochaete chrysosporium* are reported as producers of β-glucosidase (Riou *et al*., 1998; Tsukada *et al*., 2006; Gunata and Vallier, 1999) in SmF or SSF. Table 1.3 is a summary of some fungal strains involved in β-glucosidase production and the bioprocess for their production. Pre-treated biomass and pure forms of cellulose have been used as carbon sources to produce β-glucosidase with desired properties (Singhania *et al*., 2013; Rani *et al*., 2014).

Table 1.3: A summary of fungal strains producing β-glucosidases (Singhania *et al*., 2013)

Microorganism	β-glucosidase units/properties	Carbon source	Production process
Debaryomyces pseudopolymorphus	Glucose tolerant, acidic, exocellular	Cellobiose	Shake flask fermentation
Trichoderma atroviride	2.5 IU/g	Pretreated willow	Shake flask fermentation
Penicillium pinophillum	83 U/mg protein, Vmax = 1120 U/mg protein, Km = 5.5 mM, Ki = 26.6 mM, extracellular, acidic	Cellulose	SmF, Small fermenter with working volume 200 ml
Penicillium citrinum	159.1 U/g solid, Vmax = 85.93 U/mg, Km = 1.2 mM, Ki = 17.9 mM with pNPG, thermoacidophillic	Rice bran	Solid-state fermentation
Periconia sp.	Thermotolerant, active at wide pH range	Cellulose	SmF
Penicillium decumbens	Thrmotolerant, acidic BGL, Km = 0.006 mM towards pNPG	Wheat bran	SmF
Penicillium echinulatum	0.85 U/mg of protein with pNPG	Microcrystalline cellulose	SmF
Stachybotrys sp.	Km = 0.22 mM towards pNPG and 2.22 mM for cellobiose, active at 50 ^{o}C and pH = 5	Cellulose	Fed-batch SmF
Humicola isolens	BGL active at 50 ^{o}C and at pH 6.0, BGL activity was stimulated at 400 mM conc.	Microcrystalline	Shake flask
Fomitopsis palustris	Acidic BGL active at 55 ^{o}C, Km = 0.706 and 0.971 mM for pNPG of BGL1 and BGL2 respectively	Cellobiose	Shake flask
Fomitopsis sp	53 U/g	Wheat bran	SSF
Aspergillus niger	Thermostable, acidic, glucose resistant showing 92% activity retension at 250 mM glucose	Lactose, wheat bran	SmF, shake flask

SmF: submerged fermentation; SSF: solid state fermentation; BGL: β-glucosidase; Km: Michaelis Menten kinetics; Ki: Inhibition constant; Vmax: Maximum velocity; pNPG: p-nitrophenyl- β-D-glucopyranoside

1.2.9 Protein expression systems

Protein expression systems include *Escherichia coli* (*E. coli*), yeast; baculovirus infected insect cells and mammalian cells (Fernandez and Hoeffler, 1999). Table 1.4 outlines the characteristics of these expression systems (adapted from Fernandez and Hoeffler, 1999).

Table 1.4: Comparison of expression systems cells (Fernandez and Hoeffler, 1999).

Characteristics	*E. coli*	Yeast	Insect cells	Mammalian cells
Cell growth	rapid (30 min)	rapid (90 min)	slow (18-24 h)	slow (24 h)
Complexity of growth medium	Minimum	Minimum	Complex	Complex
Cost of growth medium	Low	Low	High	High
Expression level	High	Low – High	low - high	low - moderate
Extracellular expression	Secretion to periplasm	Secretion to medium	Secretion to medium	Secretion to medium
Protein folding	Refolding usually required	Refolding may be required	Proper folding	Proper folding
N-linked glycosylation	None	High mannose	Simple, no sialic acid	Complex
O-linked glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	yes
gamma-Carboxylation	No	No	No	yes

Saccharomyces cerevisiae (*S. cerevisiae*) and the methylotrophic yeast *Pichia pastoris* (*P. pastoris*) are commonly used yeasts for protein expression with commercial cloning and expression kits readily available (EasySelect™ *Pichia* Expression Kit user guide, 2010).

1.2.10 *Pichia pastoris* expression system

P. pastoris is a methylotrophic yeast with a successful system for production of recombinant proteins. It is similar to *S. cerevisiae* in general growth conditions and handling (EasySelect™ *Pichia* Expression Kit user guide, 2010). There are several factors that have contributed to the rapid acceptance of *P. pastoris* as an expression system:

i. Similarity with *S. cerevisiae* in terms of techniques needed for genetic manipulation,

ii. It has a unique promoter derived from the alcohol oxidase 1 gene (*AOX1*) suited for the controlled expression of foreign genes,

iii. It is faster, easier and less expensive to use than other eukaryotic expression systems.

iv. As a eukaryote, it has the advantage of higher eukaryotic expression systems such as protein processing, protein folding and post-translational modification.

P. pastoris is capable of metabolizing methanol as its sole carbon source and the first step involves oxidation of methanol (CH_3OH) to formaldehyde (HCOH) by the enzyme alcohol oxidase, using molecular oxygen (Cereghino and Cregg, 2000; Figure 1.5). The methanol-inducible promoter (*AOX1*) has been widely used for controlled expression of eukaryotic proteins.

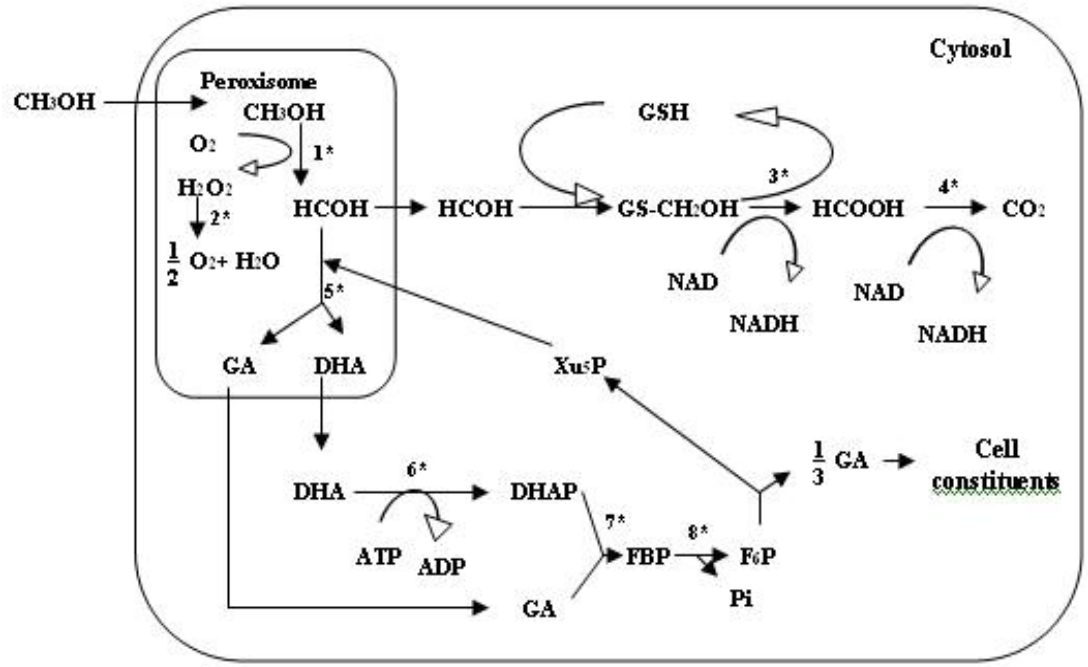


Figure 1.5: The methanol pathway in *P. pastoris*. (1) alcohol oxidase; (2) catalase; (3) formaldehyde dehydrogenase; (4) formate dehydrogenase; (5) dihydroxyacetone synthase; (6) dihydroxyacetone kinase; (7) fructose 1,6-biphosphate aldolase; (8) fructose 1,6-bisphosphatase (Cereghino and Cregg, 2000).

The genotypic and phenotypic characteristics of *P. pastoris* strains used for expression of foreign proteins are summarized in Table 1.5.

Table 1.5: Genotype and phenotype of *P. pastoris* strains

Strain	Genotype	Phenotype (*Pichia* only)	Application
X-33	wild-type	Mut+	Selection of Zeocin resistant expression vectors
GS115	*his4*	His–, Mut+	Selection of expression vectors containing *HIS4*
KM71H	*arg4 aox1::ARG4*	Mut^S, Arg+	Selection of expression vectors containing *HIS4* to generate strains with Mut^S phenotype

Adapted from EasySelect™ Pichia Expression Kit user guide, 2010

Wild-type X-33 or GS115 (*his4*) are commonly used expression hosts. They retain both *AOX1* and *AOX2* genes and grow on methanol (methanol utilization plus). X-33 is useful for selection on Zeocin and large scale growth. GS115 has a mutation in the histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine and therefore it will only grow on media supplemented with histidine. The KM71H strain however has a mutation in the argininosuccinate lyase gene (*arg4*) and will only grow in media supplemented with arginine (EasySelect^{TM} Pichia Expression Kit user guide, 2010).

1.2.11 Bacterial cellulose

Although plants are considered to be the major producers of cellulose, certain bacteria such as *Gluconacetobacter xylinus* (*G. xylinus*) are also known to produce cellulose (Romling, 2002). Bacterial cellulose (BC) produced by *G. xylinus* is synthesized as an extracellular gelatinous mat and the structure is an unbranched polymer of β-(1 -4) – linked glucose residues (Yadav *et al*., 2010). Apart from *G. xylinus*, other bacterial genera such as *Aerobacter*, *Alcaligenes*, *Achromobacter*, *Agrobacterium*, *Rhizobium*, *Sarcina* and *Zooglea* have been reported to have BC synthesizing properties (Jonas and Farah, 1998; Matthysse *et al*., 1995; Ross *et al*., 1991). *Gluconacetobacter* species are reported to be the most efficient producers of BC (Bielecki *et al*., 2005).

The mechanism of BC synthesis involves two segments. The first part involves the synthesis of uridine diphosphoglucose (UDPGIc) which begins with carbon compounds such as hexoses and glycerol to produce UDPGIc, while the second mechanism involves the polymerization of glucose into β- 1→4 glucan unbranched chains and it is thought to involve lipid intermediates (Delmerl and Amor, 1995; Iannino *et al*., 1998).

BC is formed in the membranes of *G. xylinus* by a cellulose synthase complex and secreted from a row of 80 pore-like sites along the axis of the cell from which the microfibrils join to create ribbon of cellulose (Figure 1.6) (Amano *et al*., 2005; Ross *et al*., 1991; Keshk,

2014). The result of further interaction of microfibrils is a dense interlocking mesh of cellulose microfibrils to form a layer of cellulose which floats on aqueous substrate. Unlike plant cellulose, cellulose produced by *Gluconacetobacter* strain is devoid of other contaminating polysaccharides and its isolation and purification are relatively simple, not requiring energy- or chemical-intensive processes (Keshk, 2014). This property makes production of cellulose from bacteria to be of potential economic interest.

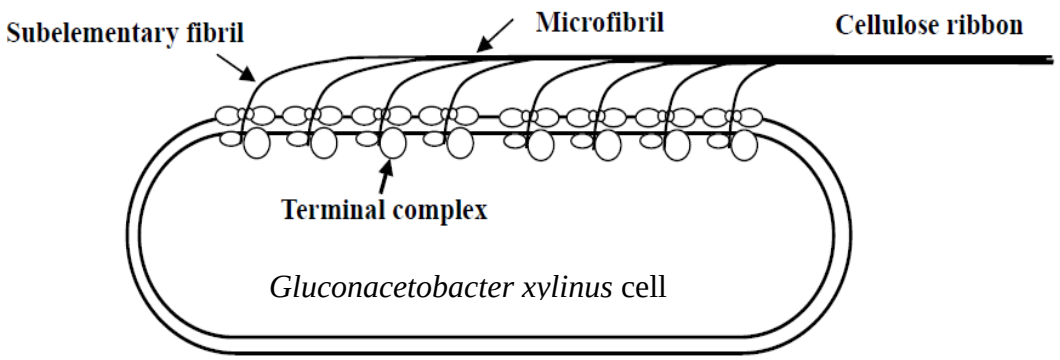


Figure 1.6: Mechanism for cellulose ribbon formation by the aggregation of cellulose chains extruded from a *Gluconacetobacter* cell (Amano *et al*., 2005)

Synthetic polymers, especially packaging materials, are produced from fossil resources. Consumption of these plastic packaging materials contributes to waste accumulation in the environment particularly in developing countries. There is therefore a need for alternative raw materials which should be biodegradable. BC is a renewable natural nanomaterial with mechanical properties, moldability porosity, and water absorbency and is biodegradable (Hu *et al*., 2014). These properties suggest BC to have a wide application in the food industry, paper industry, cosmetic and fashion industry, refineries, pharmaceutical industry and other fields (Keshk, 2002; Chawla *et al*., 2009; Budhiono *et al*., 1999; Fu *et al*., 2013). In addition to adhesive properties, BC is reported to confer UV protection, ensures

maintenance of an aerobic environment, retains moisture and protects against heavy metal stress, a property which has been widely explored for various medical applications (Rajwade *et al*., 2015).

The accumulation of industrial and agricultural waste residues causes degradation of the human environment, constitutes a health hazard due to both direct pollution and the support for the growth of microorganisms, while also causing huge losses of valuable constituents which when processed could yield food, chemicals and fuel (Saranraj *et al*., 2012). Previous studies have illustrated that BC can be produced from agricultural residues such as wheat straw and cotton-based textile waste (Cavka *et al*., 2013). Cavka *et al*., (2013) demonstrated that waste fibre sludge is a suitable raw material for the production of BC and enzymes through sequential fermentation. The use of industrial waste or agricultural residues as feedstock for the production of BC has advantages in a country like Nigeria where industrial waste and agricultural residues constitute a nuisance to the environment (Ezeonu *et al*., 2012).

1.2.12 Research hypotheses

This research work focuses on the following hypotheses:

- The *Aspergillus* genus harbours previously uncharacterised β-glucosidase genes with attractive properties for cellulose degradation systems.

- Cellulose producing bacteria such as *Gluconacetobacter xylinus* produce bacterial cellulose and β-glucosidases with novel characteristics.

- Fungal β-glucosidases require co-production of other proteins such as hydrophobins to allow access to lignocellulose which is hydrolysed to fermentable sugars.

An initial bioinformatics analysis of the genome databases (e.g. CADRE, http://www.cadre-genomes.org.uk/index.html) for β–glucosidases were collated for the variation in this enzyme class and focus upon novel candidate products for cloning and expression. Potential bacterial donors of cellulose degrading enzymes were also be explored for their expression in fungal hosts and the possibility of boosting β-glucosidase activity by co-expression with engineered hydrophobins were be examined.

1.2.13 Specific research objectives:

- Identification of candidate enzymes using genome project data.
- Preliminary screening of organisms (fungi and bacterial strains) and carbon sources for β–glucosidase production.
- Cloning and expression of β-glucosidases and hydrophobins.
- Biochemical characterization of the candidate β–glucosidases and hydrophobins using both rapid plate assays and detailed biochemical measurement.

Chapter 2

General materials and methods

2.1 Database search

The following databases/programs were used in this study: *Aspergillus* comparative database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/Multihome.html), CADRE (Central *Aspergillus* Data Repository; (http://www.cadre-genomes.org.uk/index.html) and the National Centre for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/). Multiple Sequence Alignments (MSA) were performed to identify regions of similarity, structure and function between sequences with TCoffee advanced (Notredame *et al*., 2000), SignalP 4.1 (to avoid similarities within the signal sequence alone) and ClustalX 2.1 (http://www.clustal.org/clustal2/) (Page, 1996).

2.2 Organisms

All the fungi (Table 2.1) and bacteria (Table 2.2) used in this study were obtained from the University of Wolverhampton culture collection centre except for the Nigerian and Polish strains of *A. niger* F321 and *A. niger* F320 (Table 2.1). Four-well characterized *A. nidulans* strains were also included as controls (Table 2.1). The cultures were chosen as a variety of sources predicted with cellulose degrading activity (e.g. Edwards *et al*., 1998; O'Neil *et al*., 2002). Five *Pichia* clones carrying *A. nidulans* clones in pPICZ vectors that exhibited satisfactory levels of expression of recombinant β-glucosidase (Bauer *et al*., 2006) were obtained from the Fungal Genetics Stock Centre (FGSC) USA (Table 2.3). *Pichia pastoris* 323 strain which served as a control, was obtained from the University culture collection center. *Aspergillus* strains were grown on Malt Extract Agar (per litre distilled water: malt extract – 30 g, agar – 15 g, pH 5.5 ± 0.2) at 30^{o}C and then stored at 4^{o}C.

Table 2.1: *Aspergillus* and *Dendryphiella* strains used in this study

Species	UoW identification No.	Strain	Genotype	Source
Aspergillus niger	F7	-	-	Unknown
Aspergillus niger	F9	PROM 2	-	Dr. M. Whitehead
Aspergillus niger	F10	PROM 4	-	Dr. M. Whitehead
**Dendryphiella arenaria*	F200	4491	-	Malaysia, 1989
* *Dendryphiella salina*	F201	0530	-	Unknown
* *Dendryphiella salina*	F202	0538	-	Southsea, UK. 1960
* *Dendryphiella salina*	F203	3564	-	Southsea, UK. 1960
* *Dendryphiella salina*	F204	5523	-	Hayling Island, UK. 1991
* *Dendryphiella arenaria*	F205	4493	-	Unknown
* *Dendryphiella arenaria*	F206	4494	-	Malaysia, 1989
* *Dendryphiella arenaria*	F207	4495	-	Malaysia, 1989
* *Dendryphiella arenaria*	F208	4497	-	Unknown
* *Dendryphiella salina*	F10	4519	-	Unknown
Aspergillus niger	F212	0532	-	Unknown
Aspergillus niger	F287	-	-	IMIO 17454, 2004
Aspergillus niger	F320	-	-	Poland, 2012
Aspergillus niger	F321	-	-	Soil, Nigeria, 2012
[+]*Aspergillus nidulans*	F3	L.19	*yA2, pyro A4*	Dr. P. Hooley
[+]*Aspergillus nidulans*	F4	L.20	*wA3, paba A1*	Dr. P. Hooley
[+]*Aspergillus nidulans*	F213	POL-1	*biA1, aga A50*	Poland
[+]*Aspergillus nidulans*	F199	GO281	*yA2, paba A1, sltA1*	University of Glasgow

UoW: University of Wolverhampton; [+]O'Neil *et al*., 2002; *Edwards *et al*., 1998

Table 2.2: Bacterial strains used in this study

Species	UoW identification No
B. cereus	10
B. thuringiensis	12
B. circulans	13
B. megaterium	16
B. pumilus	18
B. stearothermophilus	19
B. subtilis	20
B. sphaericus	87
Gluconacetobacter xylinus	639
Escherichia coli JM109	Sigma-Aldrich

UoW: University of Wolverhampton.

Table 2.3: *Pichia pastoris* clones carrying *A. nidulans* β-glucosidase gene (Bauer *et al*., 2006)

Accession No.	FGSC Identification No.	UoW identification No
AN2612.2	10081	WU644
AN0712.2	10063	WU640
AN1551.2	10069	WU641
AN2227.2	10075	WU643
AN1804.2	10072	WU642

FGSC: Fungal Genetic Stock Center, UoW: University of Wolverhampton

These *P. pastoris* strains are subsequently referred to using the acronym of the *A. nidulans* gene contained in the expression cassette.

2.3 Rapid method for screening of organisms for cellulase activity

A simple, rapid and sensitive cellulase assay procedure based on the Congo red clearing zone assay method by Sazci *et al*., (1986) was used for screening. This method allows the simultaneous enzyme comparison for many samples and has been developed to allow the detection of β-glucosidase activity under a wide range of conditions for a spectrum of strains. The chemicals used in this study are described in Appendix 1.

Aspergillus strains were initially grown on Malt Extract Agar (MEA - Malt extract - 30 g, agar - 15 g, per liter) plates while bacterial strains were grown on Tryptone Soya Agar (TSA - Casein peptone (pancreatic) – 15 g, Soya peptone (papainic) – 5 g, Sodium chloride – 5g, agar – 15 g, per litre, Final pH 7.3). Cultures were stored at 4^{o}C. For *Aspergillus* strains (Table 2.1), spores were aseptically picked from plates and inoculated into 5 ml sterile nutrient broth in a McCartney bottle. The bottles were incubated at 30^{o}C in an orbital shaker (Model G25, S/No. 390534557 U/K) at 150 rev/min for 2 days. Sterile petri dishes were prepared and each Petri dish was made up of 15 ml CMC (Carboxymethylcellulose) agar, made with 0.5 g CMC (a soluble form of cellulose); 0.1 g $NaNo_3$; 0.1 g K_2HPO_4; 0.1 g KCl; 0.05 g $MgSO_4$; 0.05 g yeast extract; and 0.1 g glucose in 100 ml of distilled water. The medium was solidified using 1.7% w/v agar no 2. To each plate, 0.2 ml of the nutrient broth culture was placed in the centre of the Petri dish and incubation carried out at 5^{o}C intervals in the range of 25 - 50^{o}C for 6 days. For the control, nutrient broth without the culture was used. After incubation each plate was flooded with 0.1% Congo red solution, and shaken at 50 rev/min for 15 minutes on a shaker (Model R100 rotatest shaker Luckham, S/No. R/2268/B). The plate was also de-stained (flooded) with 1 M NaCl salt solution by shaking again at 50 rev/min for 10 – 15 minutes. The NaCl solution elutes the dye in the clearing zone where the cellulose has been degraded into simple sugars by the enzymatic activity. Microbial growth was stopped by flooding the

plates with 1 M NaOH (pH 13.3) which slightly changed the dye color to brownish-red and also inhibited the enzyme activity. The diameter of the clear zone was measured to provide quantitative cellulolytic activity. The assay was carried out in triplicate for each sample and three diameters were measured per plate.

For bacterial strains (Table 2.2), colonies were aseptically picked from TSA plates and inoculated into 5 ml sterile distilled water in a McCartney bottle. The bottles were incubated at 30^{o}C in an orbital incubator shaker (Model G25, S/No. 390534557 U/K) at 150 RPM for 1 day. To each CMC agar plate, 40 µl of the culture was placed and sterile distilled water without the culture was used as control. The assay was carried out as outlined above using the Congo red assay method, but the incubation period was for 2 days.

2.4 Yeast Extract Peptone Dextrose (YEPD) and Buffered Methanol-complex Medium (BMMY) media

All media were prepared according to the Invitrogen manuals (Cat. no. V195-20, Cat. no. K1710-01 and *P. pastoris* Fermentation Process Guidelines, 2010) and were sterilized by autoclave on liquid cycle at 121 ^{o}C for 15 minutes. Recipes are described in Appendix 2.

2.5 Determination of cell growth (CFU/ml)

The colony forming unit (CFU) counts were carried out using the Miles and Misra method (Miles *et al*., 1938). Eight tubes of 4.5 ml Ringers were labeled from 10^{-1} to 10^{-8}. Using a 200 – 1000 µl Finn pipette, 500 µl of the test suspension was aseptically added into tube labeled 10^{-1}. Using a fresh tip, the content of the 10^{-1} tube was aspirated five times and 500 µl was aseptically transferred to a tube labeled 10^{-2}. In a similar way, the dilution series was completed up to and including 10^{-8}. MEA plates were marked into 8 sectors on the base, labeled 10^{-1} to 10^{-8} with name, incubation temperature, strain and date. Starting with the most dilute (10^{-8}), 20 µl of the suspension was transferred onto the sector labeled 10^{-8}

in triplicate. This was done for each dilution using a fresh tip and after droplets had been soaked into the agar, the plates were incubated for 2 days at 30^{o}C and the number of countable colonies growing in each of the inoculated areas was recorded.

The dilution that gave the largest number of countable colonies was selected for the estimation of the number of viable organisms in each broth culture. For example,

Volume of drop dispensed onto 10^{-5} sector = 20 µl = 0.02 ml

No. of colonies in 1 ml of 10^{-5} dilution = 57 (colony count) X 50

No. of colonies in 1 ml (CFU/ml) of undiluted suspension = 57 X 50 X 10^{5} = 2.8 X 10^{8}

Log_{10} = 8.45 CFU/ml

NOTE: 57 = Number of colonies in 20 µl; 50 = 1/20 µl (0.02 ml)

= 50 (Number of colonies in 1 ml); 10^{5} = Dilution factor

To calculate the geometric mean (GM) of the cfu counts:

$$GM = (s1 \times s2 \times s3)^{1/N}$$

Where: **s** = no of colonies per ml for sample 1, 2, 3; **N** = no of sample

For e.g. if cfu/ml is 8.45, 8.45, 8.45

The GM is determined by taking the 3rd root of the product of the 3rd readings:

$$GM = (8.45 \times 8.45 \times 8.45)^{1/3} = 6.83 \text{ cfu/ml}$$

All results were statistically analysed with GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

N.B. - More specific methods are given in the individual chapters.

Chapter 3

Bioinformatics analysis of *Aspergillus* β-glucosidase and hydrophobin genes

3.1 Introduction

3.1.1 Cellulose degradation

The degradation of cellulose involves the action of three hydrolytic enzymes, endoglucanase, exoglucanase and β-glucosidase which work in harmony to hydrolyse cellulose to fermentable sugars. These enzymes are divided into eight glycosyl hydrolase families (Vlasenko *et al*., 2010). Glycosyl hydrolases (GH) have been defined by Carbohydrate Active Enzymes (CAZy) as a group of enzymes that hydrolyse the glycosidic linkages between carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Based on their amino acid sequences, the enzymes are arranged into different families (www.cazy.org). CAZy classification of GH has placed β-glucosidase in family GH1 and GH3 based on their amino acid sequences and both families hydrolyse β-1,4-glycosidic bonds using the retaining mechanism (Henrissat, 1991; Mehdi *et al*., 2009). The GH1 family catalyses substrates following the β-retaining mechanism that employs glutamic acid as a catalytic nucleophile, while the GH3 family uses an aspartic acid residue in their nucleophile attack of substrates (Lima *et al*., 2013). The hydrolysis of cellulose glycosidic linkages by cellulase enzymes is by either retention or inversion mechanisms, utilizing two amino acid residues of the enzyme, a general acid (proton donor) or a nucleophile/base (Davies and Henrissat, 1995). During the course of this work, a paper by Lima *et al.* (2013) gave a critical insight into the structure and function of GH3 β-glucosidase and for the first time showed key domains by comparing fungal and bacterial enzymes.

Among the first published *Aspergillus* genome sequences from a group of related filamentous fungi are the genome sequences of *A. nidulans*, *A. oryzae*, *A. niger*, *A. fumigatus*, *A. terreus*, *A. clavatus*, *A. parasiticus* and *Neosartorya fischeri* (Jones, 2007; Anderson and Nielson, 2009; Galagan *et al*., 2003). Galagan *et al*., (2005) reported the genome sequence of the model organism *Aspergillus nidulans* by comparative analysis with *A. fumigatus* and *A. oryzae*. The analysis of their results showed sequence conservation of over 5000 non-coding regions actively conserved across all the three

species with protein coding genes for *A. nidulans*, *A. fumigatus* and *A. oryzae* of 9,396, 9,009 and 12,074 respectively. Although the three aspergilli differ considerably in their genome sequences, they were predicted to share an average of 68% amino acid identity. *A. fumigatus* and *A. oryzae* share 70% amino acid identity and each has 66 – 67% identity with *A. nidulans*.

Coutinho *et al*., (2009) reported three GH1 β-glucosidases and twenty one GH3 β-glucosidases from *A. nidulans* FGSC A4. They also highlighted the differences between *A. nidulans*, *A. niger* and *A. oryzae* by comparing the presence or absence of putative regulatory elements in the promoters of orthologues. Manual verification of their results indicated that 28.4% of the *A. nidulans* ORF's do not contain a secretion signal, of which 40% may be secreted through a non-classical method. Coutinho *et al*., (2009) also described *A. nidulans*, *A. niger* and *A. oryzae* to have 24, 20 and 26 GH1/GH3 enzymes respectively for each species. Although the functional prediction of CAZy is difficult because of low sequence identity, Busk and Lange (2013) reported new alignment independent techniques to recognize genes for GH1 and GH3 families and to also identify related sequences in highly different proteins.

3.1.2 Hydrophobins and cellulose degradation

A number of other accessory proteins are required for efficient cellulose degradation. Delmas *et al*., (2012) showed that in the presence of a carbon source such as wheat straw, hydrophobin encoding genes were greatly induced in *Aspergillus niger*, suggesting the potential application of hydrophobins in lignocellulose degradation for biofuel production. Hydrophobins are surface active proteins that have very special properties in fungal growth as structural components and in the interaction of fungi and their environment (Linder *et al*., 2005). Hydrophobins are amphipathic molecules with distinct hydrophobic and hydrophilic ends. The hydrophobic end may bind to hydrophobic residues in lignocellulose so exposing a hydrophilic site to allow cellulose access. Mandels and Reese (1964)

introduced the $C_1 - C_x$ model and hypothesized that C_1 (which is an unknown component of the cellulase system) opens up the cellulose matrix allowing the accessibility of the hydrolytic enzymes (C_x) to hydrolyse cellulose (Arantes and Saddler, 2010). Hydrophobins could play this important role of C_1. The self-assembling property of hydrophobins at hydrophilic-hydrophobic interfaces enables the formation of amphipathic membranes which changes the surface hydrophilic or hydrophobic nature of their target surfaces (Wang *et al*., 2011) and by so doing, hydrophobins help to produce aerial structures (Talbot, 1999). Attachment of hydrophobins to lignocellulose may induce surface modifications thereby hydrolytically loosening or disrupting the fibril network. Because of their special properties, hydrophobins have received attention for different applications in industries (Linder *et al*., 2005; Hektor and Scholtmeijer, 2005).

Based on their hydropathy patterns and monomer and assembled interface solubility characteristics, hydrophobins were originally classified into two classes, Class I and class II (Littlejohn *et al*., 2012). Class I and class II hydrophobins also have different amino acid sequence arrangements but all are based around four pairs of cysteine residues (Wessels, 1997). Class I and class II hydrophobin have a general Cys-patterns as follows:

Class I: $X\text{--}C\text{-}X_{5\text{-}7}\text{-}C\text{-}C\text{-}X_{19\text{-}39}\text{-}C\text{-}X_{8\text{-}23}\text{-}C\text{-}X_{5}\text{-}C\text{-}C\text{-}X_{6\text{-}18}\text{-}C\text{-}X_{2\text{-}13}$

Class II: $X\text{--}C\text{-}X_{9\text{-}10}\text{-}C\text{-}C\text{-}X_{11}\text{-}C\text{-}X_{16}\text{-}C\text{-}X_{8\text{-}9}\text{-}C\text{-}C\text{-}X_{10}\text{-}C\text{-}X_{6\text{-}7}$

Where X = any amino acids while C = cysteine (Kershaw and Talbot, 1998; Littlejohn *et al*., 2012).

Yaguchi *et al*., (1993) and De Vries *et al*., (1993) have suggested that the cysteine residues in class I hydrophobin and class II hydrophobin sequences are involved in the formation of four disulphide bridges.

3.1.3 Bioinformatics approaches to gene identification

Gene prediction involves the process of identifying the regions of genomic DNA that encode genes, protein coding genes and prediction of functional elements such as regulatory regions (Yandell and Ence, 2012; Campbell and Heyer, 2003; Baldi and Brunak, 1998). The benefits of bioinformatics approaches in gene identification include a greater understanding of genome structure and evolution, insights into gene regulation and the discovery of novel enzymes for biotechnological application (Jones, 2007). Local alignment algorithm techniques such as BLAST, ClustalW and TCoffee look for regions of similarity between the target genes and their products. Some of the algorithms that are used in gene finding are summarized in Table 3.1, including a brief description of why they are used and their web addresses (URL's).

3.1.4 Aims and hypothesis

The aim of this chapter is to characterize the β-glucosidase status of all available members of the *Aspergillus* genus (Hakkinen *et al*., 2012) and also categorize selected hydrophobin genes in *Aspergillus nidulans* using bioinformatics tools. Ideal β-glucosidase enzyme candidates for heterologous expression will be identified based on short length and predicted properties such as acid and thermo-tolerance. This will help in the selection of β-glucosidases and hydrophobins with novel properties which will be used for expression and design of enzyme cocktails used for cellulose hydrolysis.

3.2 Materials and methods

3.2.1 Keyword searches

The *Aspergillus* comparative database (Arnaud, *et al*., 2010) was initially searched using the keyword "β-glucosidase". A BLASTp, Basic Local Alignment Search Tool (peptide), search in the *Aspergillus* comparative database was then conducted against each of the resulting keyword FASTA sequences. Signal sequence peptide presence was checked using SignalP 4.0. Once identified, signal sequences were manually removed from an amino acid sequence at the specified cleavage site. BLASTp search was again conducted to identify more possible β-glucosidases, as previous searches may have been biased by the similarities within the signal sequence alone.

A keyword search was also conducted on the Uniprot database. An updated list of accession numbers for the β-glucosidases was then checked individually using a BLASTp search on the *Aspergillus* comparative database. The results were then checked for amino acid length, number of introns, signal peptide, GH family and putative matches. The FungiDB database was also searched using the text search term "β-glucosidase". Individual accessions from fungi were then collated. A BLASTp search in the *Aspergillus* comparative database was then conducted against each of the resulting keyword FASTA sequences and the results were then checked for amino acid length, number of introns, signal peptide, GH family and putative matches. After the signal sequences of each protein were identified and removed using SignalP 4.0 server, a new BLASTp search was conducted on the remaining protein sequences.

Multiple sequence alignment was performed with a TCoffee (Tree-based Consistency Objective Function For Alignment Evaluation (Notredame *et al*., 2000). Table 3.1 is a summary of all the tools used in bioinformatics studies.

Table 3.1: Summary Table of Uniform Resource Locator (URL's) and software used in Bioinformatics work

Tool	URL	Purpose	Ref.
TCoffee	http://www.igscnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi	Used to identify regions of similarity, structure and function between sequences.	Notredame, *et al*., 2000
NCBI	http://www.ncbi.nlm.nih.gov/	Creates public databases, conducts research in computational biology, and develops software tools for analyzing genome data.	Altschul, *et al*., 1997
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	For comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences.	Altschul, *et al*., 1997
SignalP	http://www.cbs.dtu.dk/services/SignalP/	For identification of signal peptides and prediction of their cleavage sites.	Nielsen, *et al*., 1997
PSIPRED	http://bioinfo.cs.ucl.ac.uk/psipred/	Used to investigate protein secondary structure.	Jones, 1999
GenThreader	http://bioinfo.cs.ucl.ac.uk/psipred/	For prediction of 3D protein structure from amino acid sequence.	Jones, 1999
YASARA	http://www.yasara.com	For molecular visualization and modelling of protein.	YASARA Biosciences (2007)
Artemis	https://sanger.ac.uk/resources/software/artemis/	For visualization of sequence features.	Berriman and Rutherford, 2003
Aspergillus comparative database	http:broadinstitute.org/annotation/genome/	A curated comparative genomics resource for gene, protein and sequence information for *Aspergillus* research.	Arnaud, *et al*., 2010
Treeview	http://www.clustal.org/clustal2/	A simple program for displaying phylogenies.	Page, 1996
ClustalW	http://www.clustal.org/clustal2/	A general purpose multiple sequence alignment program for DNA or proteins. Highlights areas of similarity which may be associated with specific features that have been more highly conserved than other regions. For phylogenetic analysis.	Thompson, *et al*., 1994
CADRE	http://cadre-genomes.org.uk/index.html	A public resource for genomic data extracted from species of *Aspergillus.*	Mabey, *et al*., 2012
UNIPROT	http://ebi.ac.uk/uniprot/database/.html	A high quality annotated and non-redundant protein sequence database, which brings together experimental results, computed features and scientific conclusions.	Uniprot, 2009
FungiDB	http://fungidb.org/fungidb/	A database for functional and evolutionary comparison of fungal genomes.	Jason, *et al*., 2012

The phylogenetic trees were constructed using Treeview (Page, 1996) based on multiple sequence alignment using ClustalX with values out of 2000 bootstraps for the branch point. In the next step of the filtration procedure, selected β-glucosidases Afu6g12010 and NFIA_027390 sequences, Lima *et al*., (2013) AnBg11 and AaBg11 sequences, five *A. nidulans* recombinant β-glucosidases (Bauer, *et al*., 2006) and a *G. xylinus* β-glucosidase sequence were subjected to PSIPRED v3.3 (Psi-blast based secondary structure prediction), a popular protein structure prediction server (http://bioinfo.cs.ucl.ac.uk/psipred/; Jones, 1999; Buchan *et al*., 2013). This technique was used to investigate and identify the probable secondary structure of β-glucosidase proteins. GenTHREADER (http://bioinfo.cs.ucl.ac.uk/psipred/) and YASARA (Yet Another Scientific Artificial Reality Application; http://www.yasara.com) were also used to visualise and predict the tertiary structure of the proteins and determine their relationship with each other.

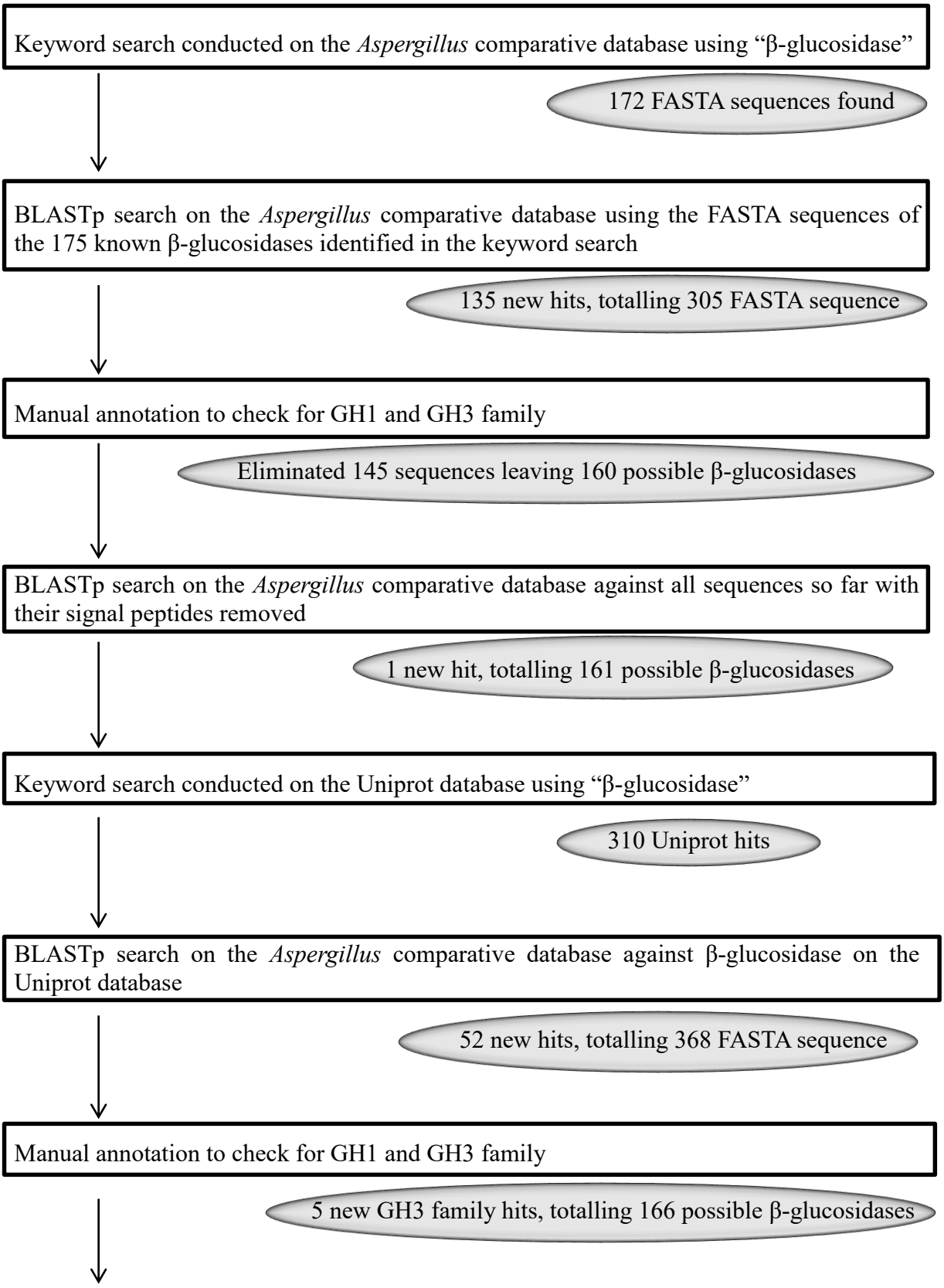
Ten hydrophobins in *A. nidulans* reported by Littlejohn *et al*., (2012) were used to show a multiple sequence alignment of the *A. nidulans* hydrophobins, highlighting the key cysteine residues. Delmas *et al*., (2012) reported two *A. niger* hydrophobins (An07g03340 and An08g09880) that are induced by lignocellulose. Two *A. niger* hydrophobins from the Delmas *et al*., (2012) report (whose signal peptides were checked using SignalP 4 and were manually removed); and nine other *A. niger* hydrophobins from Littlejohn *et al*., (2012) report were compared with the ten *A. nidulans* hydrophobins in a phylogeny.

3.3 Results

3.3.1 Screening of FASTA sequences by keyword and Blast

The keyword search using "β-glucosidase" on the main *Aspergillus* comparative database at the initial search returned 172 FASTA sequences. A text search term "β-glucosidase" on Uniprot and FungiDB database also returned 310 and 203 FASTA sequences respectively. Figure 3.1 is a summary of the screening of FASTA sequences by keyword and BLAST. Based on amino acid sequences, β-glucosidases were classified as belonging to GH1 or GH3 glycosyl hydrolase family (Henrissat, 1991). After BLASTp of all the sequences on the *Aspergillus* comparative database, they were then screened on the automatic annotation (GH1 or GH3) resulting in a total of 166 possible β-glucosidases.

Flow diagram of searches



Continuation of figure

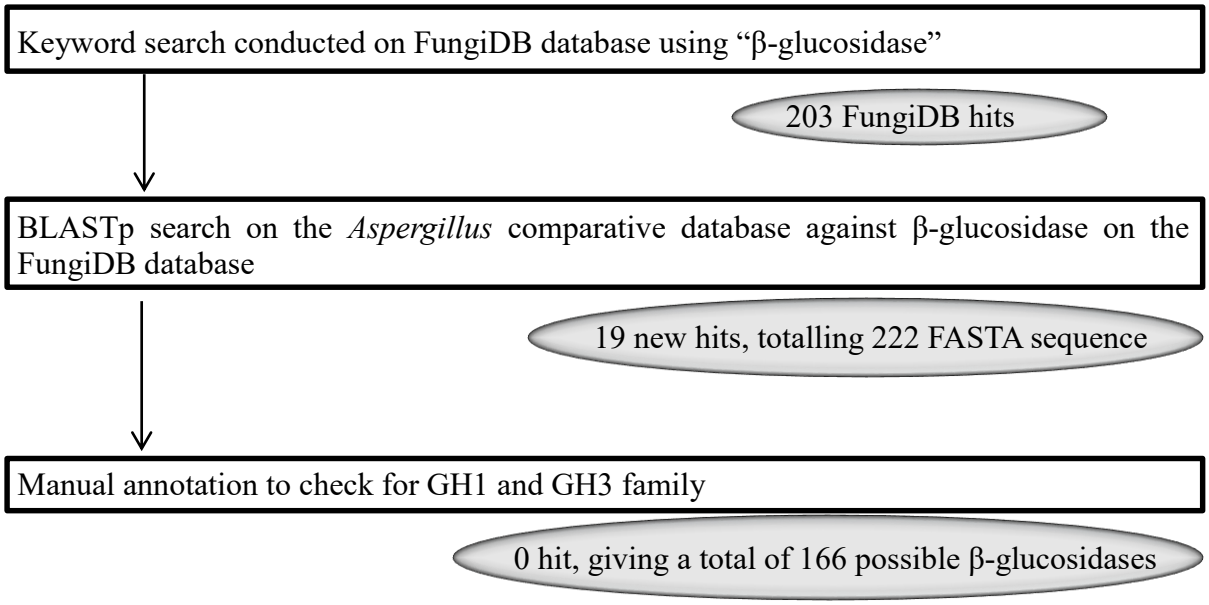


Figure 3.1: Flow diagram showing keyword search on *Aspergillus* comparative database, UNIPROT and FungiDB database

Table 3.2 is the summary of manual searches of β-glucosidase sequences from the *Aspergillus* comparative database (http://www.broadinstitute.org). Each sequence was manually checked for active sites (Lima *et al*., 2013). For more details on table, see Appendix 28.

Table 3.2: Summary of manual searches of β-glucosidases from *Aspergillus* comparative database

Species	Annotated β-glucosidase	Hypothetical β-glucosidase	GH1	GH3	Total
A. flavus	5	23	3	25	28
A. nidulans	20	3	3	20	23
A. fumigatus	14	6	5	15	20
A. terreus	2	19	3	18	21
N. fischeri	-	22	5	17	22
A. clavatus	2	10	4	8	12
A. oryzae	1	25	3	23	26
A. niger	7	7	3	11	14
Total	**51**	**115**	29	137	**166**

GH: Glycoside Hydrolase; GH1 & GH3: β-glucosidases. Hypothetical β-glucosidases are predicted to be expressed from an open reading frame making a substantial fraction of proteomes but no experimental evidence that they are expressed *in vivo* (Ijag *et al*., 2015).

From Table 3.2, 115 of the β-glucosidases are indicated to be "hypothetical" while 51 are annotated as β-glucosidases. Classification of the β-glucosidases based on GH family placed 29 β-glucosidase enzymes in the GH1 family and 137 into the GH3 family. Annotation of Carbohydrate Active enZymes (CAZy) has been done for many fungal genomes (Coutinho *et al*., 2009) and the classification can be found at www.cazy.org. Table 3.3 is a summary of GH1 and GH3 annotations from Coutinho *et al*., 2009 for comparison.

Table 3.3: Summary of GH1 and GH3 β-glucosidase annotations from Coutinho *et al*., (2009)

Species	GH1	GH3	Total
A. nidulans	3	21	24
A. niger	3	17	20
A. oryzae	3	23	26

3.3.2 Signal peptide of β-glucosidases

The identification of signal peptides in each protein was carried out using SignalP 4.1 to predict the presence and location of signal peptide cleavage sites in amino acid sequences. Seven FASTA sequences selected in Figure 3.2 along with annotated *A. niger* FASTA sequences from NCBI with accession number gi_7009581 and *A. fumigatus* FASTA sequence from CAZy with accession number gi_323473065 were subjected to SignalP 4.1 to check signal peptides. ATEG-02657, gi_323473065 and gi_7009581 have signal peptides at the cleavage site between residues 17 and 18; 19 and 20; and 19 and 20 respectively. For full details of FASTA sequences with/without signal peptide, see Appendix 28. The results indicated that some of the β-glucosidase proteins have signal peptides attached to their N-terminals which range between 15 – 27 amino acids in length

(Appendix 28). The prediction results also revealed some of the protein sequences to lack signal peptides. Fifty four percent of the accessions lacked a signal peptide, 3.96% of the accessions were without introns. The range and mean number of introns per gene was 0 - 10 and 3.31 respectively.

The signal peptides were manually removed at their specific cleavage site to avoid similarities within the signal peptide sequence alone. Multiple sequence alignment using TCoffee was performed to identify regions of similarity within the sequences. To determine the evolutionary relationship between the organisms, multiple sequence alignments using ClustalX were also performed to produce phylogenetic trees.

3.3.3 Summary of manual searches compared to Coutinho *et al*., 2009

Searches were made for fungal β-glucosidase on *Aspergillus* comparative database, Uniprot and FungiDB followed by BLASTp manual searches of the FASTA sequences on the *Aspergillus* comparative database in order to retrieve those sequences not accounted for in the respective databases. Table 3.4 is a summary of the manual searches of *A. nidulans*, *A. niger* and *A. oryzae* β-glucosidases conducted in this study compared to Coutinho *et al*., 2009.

Table 3.4: Summary of manual searches for β-glucosidases in three *Aspergillus spp*. from this study compared to the study of Coutinho *et al*., 2009

Species	This study	Coutinho *et al*., 2009
A. nidulans	23	24
A. niger	14	20
A. oryzae	26	26

3.3.4 Multiple sequence alignment of β-glucosidases

Multiple sequence alignment of the amino acid sequences was performed on the TCoffee server (http://tcoffee.crg.cat/apps/tcoffee/index.html) to highlight conserved sites and to compare putative β-glucosidases with known models. Sequences from *A. niger* (AnBg11) (Lima *et al*., 2013) were aligned with two *A. nidulans* β-glucosidases (Accession No.: AN2227.2, AN1804.2) and *Thermotoga neapolitana* (TnBgl13B) (PDB code 2X40) β-glucosidase (Table 3.5) to check for conserved residues. The two *A. nidulans* examples were chosen as representing a gene without predicted introns and one whose gene product lacked a predicted signal peptide.

Table 3.5: Examples of *Aspergillus* β-glucosidase GH3 family showing variation in the number of introns and signal peptide cleavage position

Accession No.	Species	Amino acid length (Bases)	Total No of Introns	Cleavage site (Position between)
AnBg11	*A. niger*	860	6	19 – 20
AN2227.2	*A. nidulans*	838	2	Absent
AN1804.2	*A. nidulans*	618	0	19 – 20
TnBg13B	*T. neapolitana*	721	0	Absent

A more complete list is shown in Appendix 28

A complete sequence alignment was carried out on the alignment program TCoffee (Notredame *et al*., 2000) to generate a multiple sequence alignment. The multiple sequence alignment results (Figure 3.2) revealed that there is good alignment among the β-glucosidase proteins. AnBg11 and AN1804.2 both have signal peptide cleavage at position 19/20 while AN2227.2 and TnBg13B lacked a signal peptide. Multiple sequence alignment

also revealed accession TnBg13B to have nine of the core catalytic active sites while AN2227.2 and AN1804.2 had eight each of the core catalytic active sites aligned.

The PA-14 domain, for which a carbohydrate-binding role has been reported (Yoshida *et al*., 2010) was present in both AN2227.2 and AN1804.2 sequences. Also, the N-terminal domain, which is suggested to act as a solubility enhancers for the folding C-terminal domains *in vivo* (Kim[1] *et al*., 2007), was missing in AN1804.2.


N-terminal | Linker
Linker 1 --->
β-sandwich -->
-β-sandwich → | PA-14 | β-sandwich
β-sandwich | Linker 2

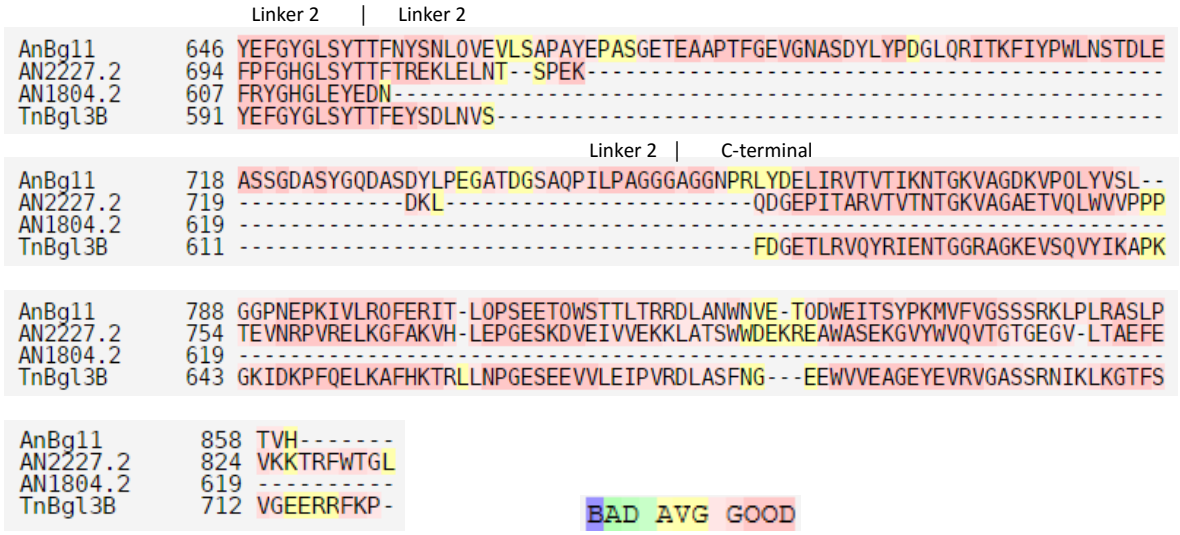


Figure 3.2: An example of a TCoffee advanced alignment. Multiple sequence alignment of β-glucosidases from *Aspergillus niger* (AnBg11), *Aspergillus nidulans* (AN2227.2, AN1804.2) and *Thermotoga neapolitana* (TnBg13B). Symbols: Catalytic site (★); helix –A and –B (green box), intermediate amino acids from linker 2 and N-terminal domain (black boxes) (Lima *et al*., 2013)

3.3.5 Phylogenetic analysis of β-glucosidases

Phylogenetic trees (neighbour joining) of the GH1 and GH3 β-glucosidase sequences were constructed using ClustalX and Treeview (Larkin *et al*., 2007) with the bootstrap value set at 2000. A value of 2000 bootstraps was chosen to calculate the robustness of each branch, indicating the number of attempts to draw a particular tree. A comparison of the phylogenetic analysis of GH1 and GH3 family (Figure 3.3) clearly shows GH1 and GH3 sequences forming separate clades. The tree drawn using GH1 and GH3 β-glucosidase sequences from different accessions revealed that the same strain of organism produces both GH1 and GH3 family β-glucosidase, for instance, ANID_06976 (belonging to GH1 family) and ANID_10372 (belonging to GH3 family) are all from *Aspergillus nidulans*.

A phylogenetic tree was also constructed to compare some selected *Aspergillus* enzymes to bacterial counterparts. *A. niger* (AnBg11) and *A. aculeatus* (AaBg11), NFIA_027390, AFL2G_06408, AO090701000841, NFIA_098520, AO090005000337, *A. nidulans* (AN2227.2, AN2612.2, AN0712.2, AN1551.2 and AN1804.2), and bacterial enzymes from *Gluconacetobacter xylinus, Acetobacterium woodii*, *Thermotoga petrophila* and *Bacillus subtilis* (Figure 3.4). Characterized bacterial enzymes were originally included in tree drawing so as to root the trees in order to identify *Aspergillus* enzymes that resembled bacterial enzymes with attractive features like thermo-tolerance. However, it became clear that examples of bacterial β-glucosidases in some cases were grouped with *Aspergillus* enzymes. Intriguingly, the *Aspergillus fumigatus* enzyme Afu6g12010 closely clustered with a *Gluconacetobacter xylinus* enzyme. A complete list of β-glucosidases FASTA sequence manual search is listed in Appendix 28. It is worthy of note that not all the GH1 and GH3 enzymes listed in the Table 3.2 have all the 10 conserved residues. Twenty eight FASTA sequences (27.72%) out of the 166 accessions lacked some of the complete active sites listed by Lima *et al*., (2013).

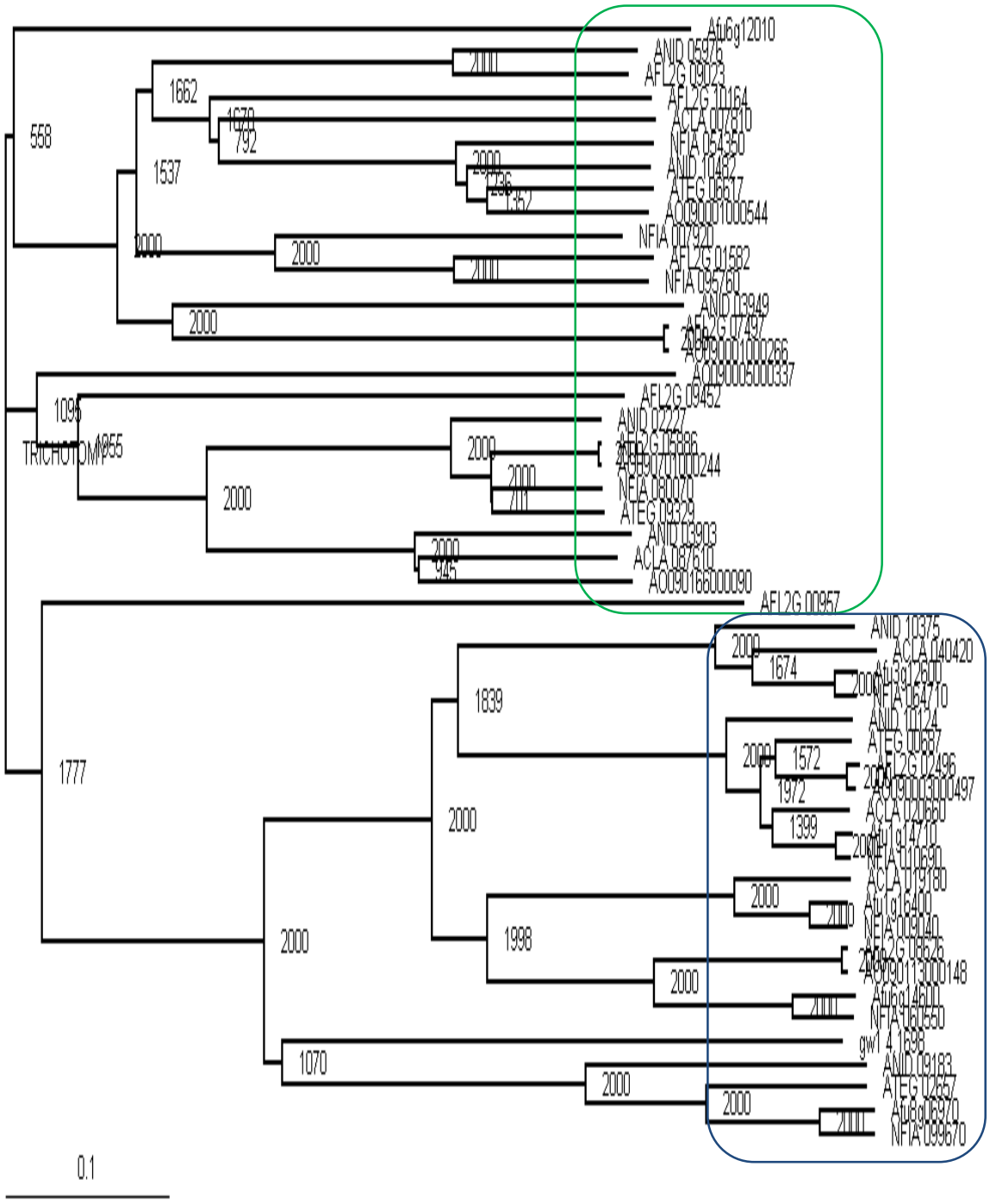


Figure 3.3: An example Treeview analysis fungal proteins (shown with values out of 2000 bootstraps for branch points). GH1 β-glucosidase family: rounded green rectangle and GH3 β-glucosidase family: rounded blue rectangle. *A. fumigatus* (Afu6g12010, Afu3g12600, Afu1g14710, Afu1g16400, Afu6g14600, Afu8g06790); *A. nidulans* (ANID_05976, ANID_10482, ANID_03949, ANID_02227, ANID_03903, ANID_10375, ANID_10124, ANID_09183); *A. flavus* (AFL2G_09023, AFL2G_10164, AFL2G_01582, AFL2G_07497, AFL2G_09452, AFL2G_05886, AFL2G_00957, AFL2G_02496, AFL2G_08626); *A. clavatus* (ACLA_007810, ACLA_087610, ACLA_040420, ACLA_020660, ACLA_019180); *N. fischeri* (NFIA_054350, NFIA_007920, NFIA_095760, NFIA_080020, NFIA_064710, NFIA_010690, NFIA_009040, NFIA_060550, NFIA_099670); *A. terreus* (ATEG_06617, ATEG_09329, ATEG_00687, ATEG_02657); *A. oryzae* (AO090001000544, AO090001000266, AO090005000337, AO090001000244, AO090166000090, AO090003000497, AO090113000148); *A. niger* (gw1.4.1698)

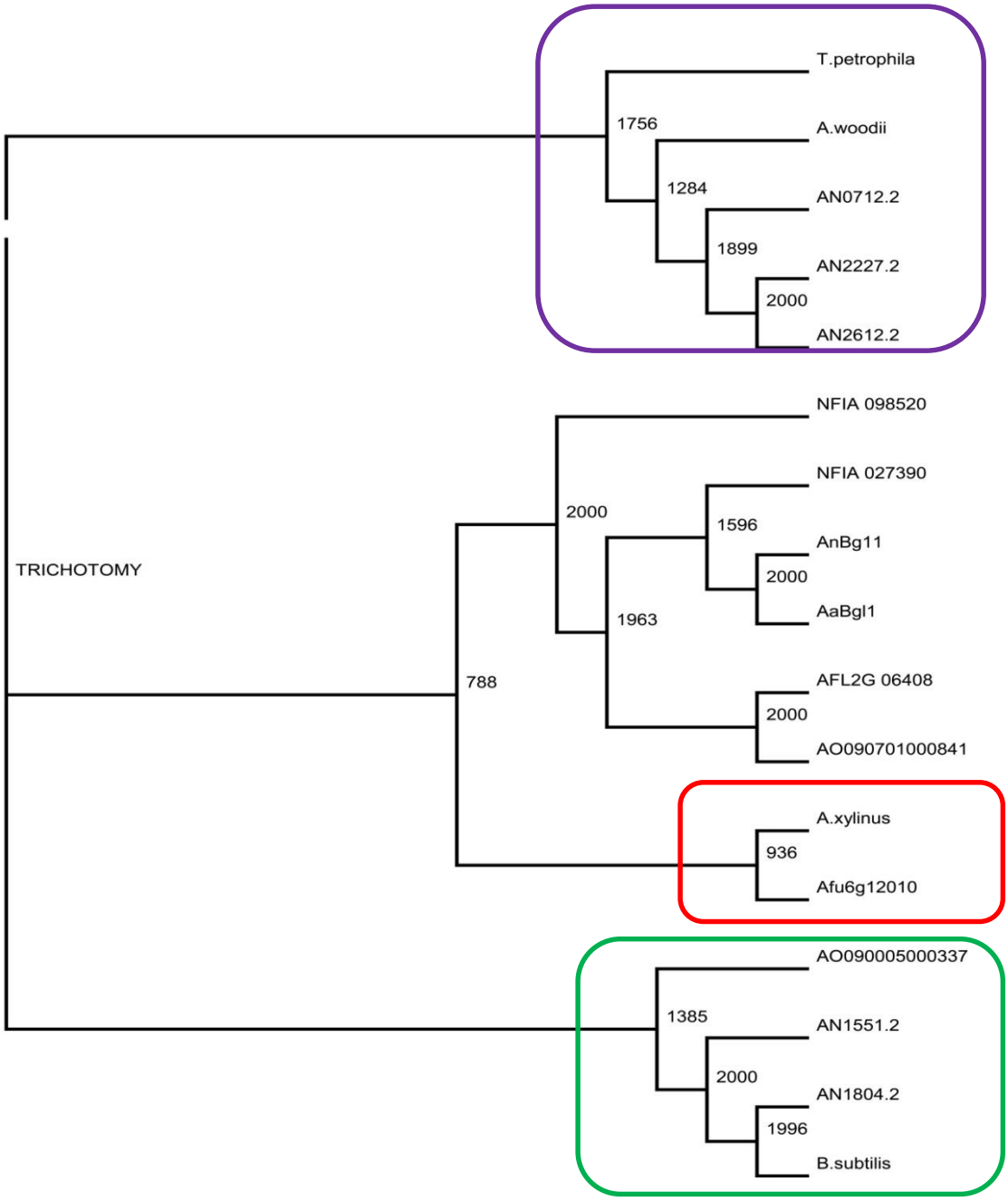


Figure 3.4: An example Treeview analysis of GH3 β-glucosidases: *A. niger* (AnBg11) and *A. aculeatus* (AaBg11), *Aspergillus fumigatus* (Afu6g12010), NFIA_027390, AFL2G_06408, AO090701000841, NFIA_098520, AO090005000337, *A. nidulans* (AN2227.2, AN2612.2, AN0712.2, AN1551.2 and AN1804.2), and bacterial enzymes from *Gluconacetobacter xylinus* (*A. xylinus*) (WP_007399076). *Acetobacterium woodii* (gi|375302290), *Thermotoga petrophila* (gi|500480046) and *Bacillus subtilis* (YP_003864539.1) (shown with values out of 2000 bootstraps for branch points). Red rounded rectangle: an example of fungal and bacterial proteins forming a clade.

3.3.6 Prediction of β-glucosidase characteristics – hydrophobicity plot

An alignMe plot (http://www.bioinfo.mpg.de/AlignMe/) (Figure 3.5) was constructed using AlignMe pairwise on β-glucosidase from fungal and bacterial sources to determine the hydrophobicity of the proteins. This gives the absolute value to each amino acid depending on its hydropathy. A negative number is hydrophilic while a positive number is hydrophobic (Littlejohn *et al*., 2012).

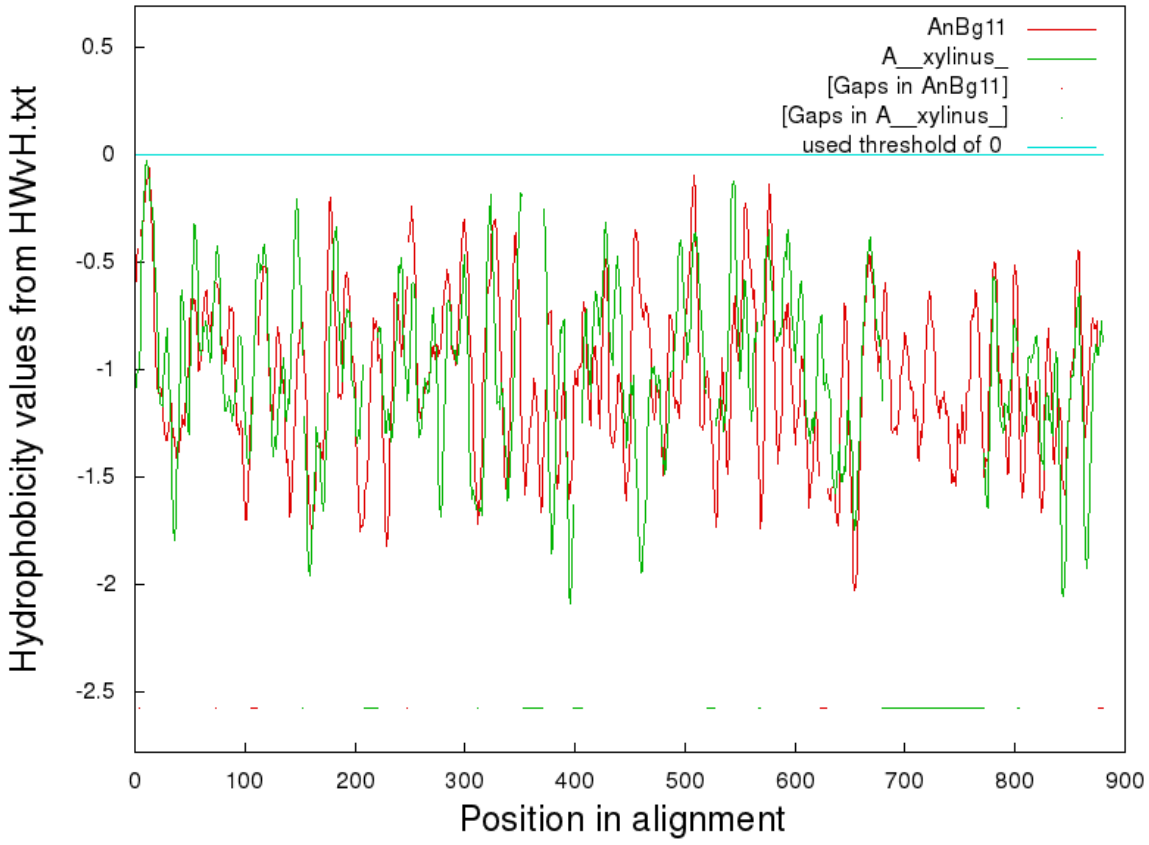


Figure 3.5: AlignMe hydrophobicity plots of β-glucosidase from *A. niger* (AnBg11) and *G. xylinus.*

Figure 3.5 shows β-glucosidases from *A. niger* and *G. xylinus* to have negative values suggesting that both proteins from the different organisms are hydrophilic. Hydrophilic groups are water loving and polar, interacting with water by hydrogen bonding. Hydrophobic groups, on the other hand, are water fearing or nonpolar, that is, unable to

interact with water. Hydrophobicity of amino acids regulates and determines where the amino acid will be located in the final structure of the protein (Kyte and Doolittle, 1982).

3.3.7 Secondary and tertiary structure predictions of β-glucosidases

PSIPRED is a protein sequence analysis workbench which incorporates two feed-forward neural networks to perform an analysis of results obtained by the Psi-blast homology search algorithm (Altschul *et al*., 1997). The selected sequences for PSIPRED analysis were put through the PSIPRED protein structure prediction server to provide an indication of their possible secondary structures (Figure 3.6). Subjective visual assessment of the secondary structure appear to show that most of the β-glucosidase sequences have similar secondary structure of coils, sheets and helices, except the clone AN1804.2 β-glucosidase structure which few beta sheets.

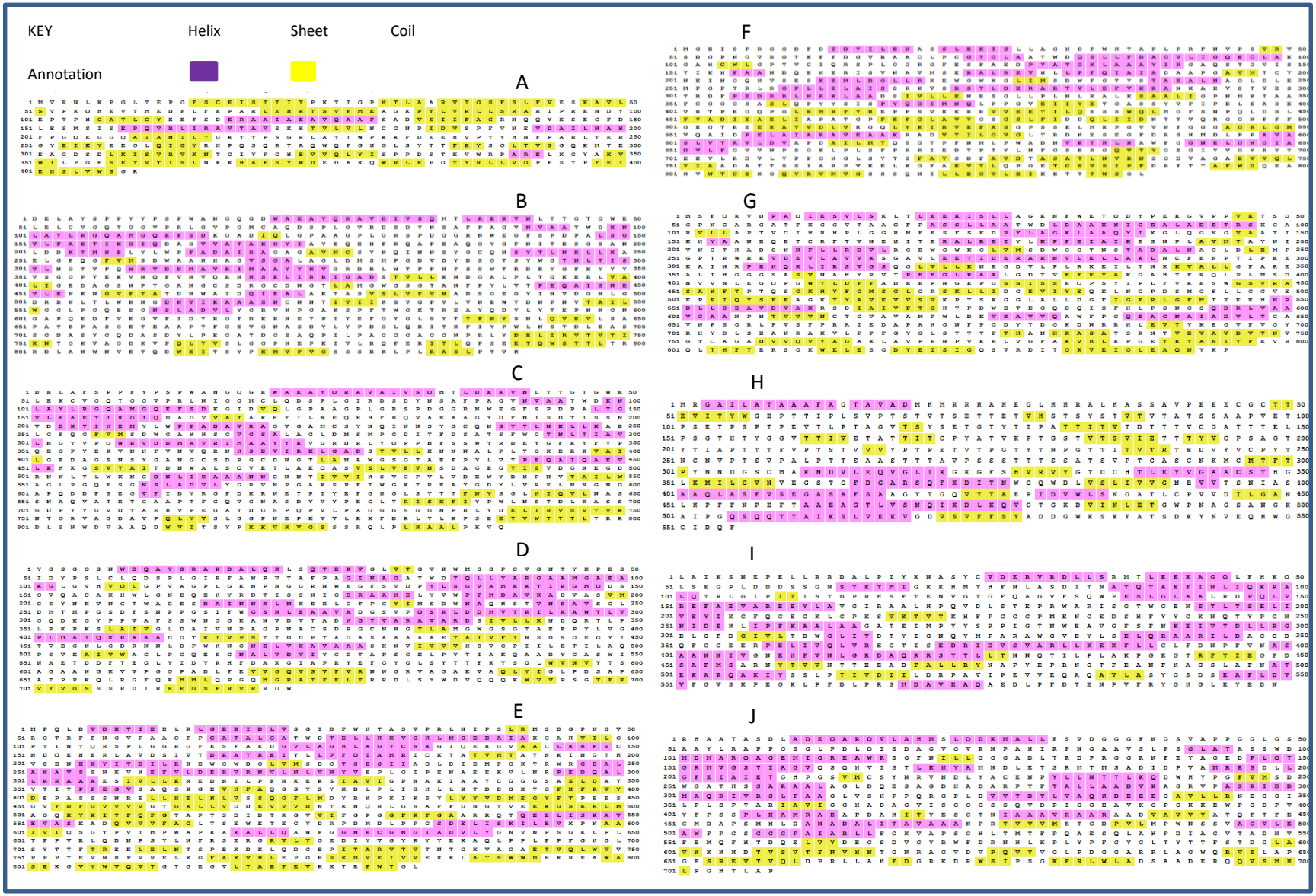


Figure 3.6: Secondary structure map analysis of selected β-glucosidase sequences. A: Afu6g12010, B: AnBg11, C: AaBgI1, D: NFIA_027390, E: AN2227.2, F: AN2612.2, G: AN0712.2, H: AN1551.2, I: AN1804.2, J: WP_014106418 (*G. xylinus*)

3.3.8 Multiple Sequence Alignments of hydrophobins

Figure 3.7 shows the multiple sequence alignment result of *A. nidulans* hydrophobin protein sequences taken from the accessions in Littlejohn *et al*., (2012). The results indicated that cysteine residues are aligned among the proteins but with little similarity outside these regions. Out of the ten hydrophobin protein sequences, only three fit into the hydrophobin cysteine pattern model of Kershaw and Talbot, (1998) and Littlejohn *et al*., (2012). Proteins with accession number ANID_01837, ANID_08006 and ANID_08803 fitted into the class I model while the other seven accessions could not fit into either model. ANID_07539 had its 5th cysteine residue after six other amino acids while ANID_05290, ANID_07327 and ANID_11982 had their 3rd cysteine residue after three other amino acids. Seven of the ten *A. nidulans* hydrophobin protein sequences had inconsistency with the model at different points by replacement of the cysteine residue with other amino acid residues. These then represent potentially novel hydrophobins of an intermediate class (Littlejohn *et al*., 2012).

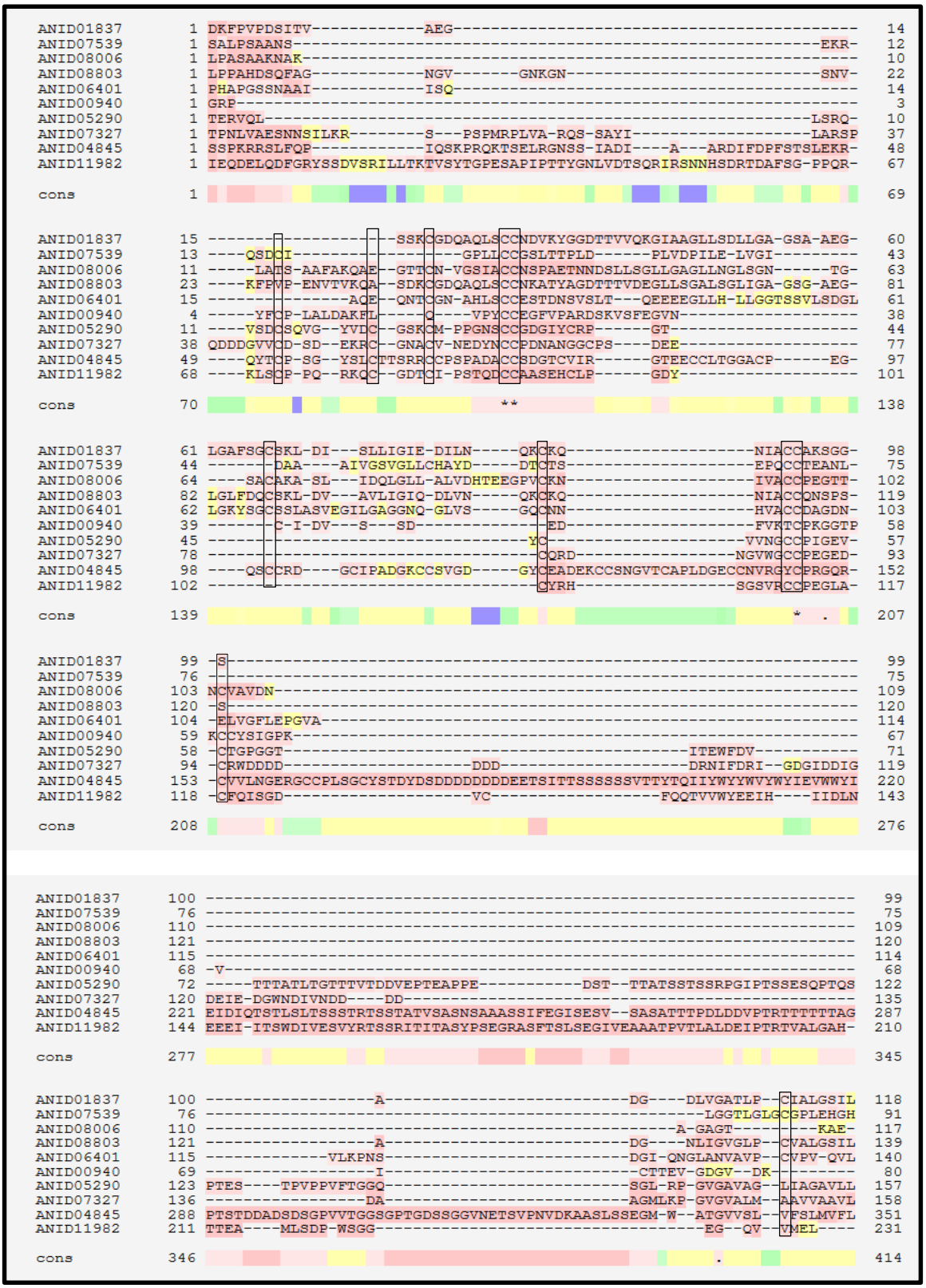


Figure 3.7: Multiple sequence alignment of *A. nidulans* hydrophobins (with their signal peptides all removed) taken from accessions listed in Littlejohn *et al*., (2012). Boxed regions highlight the key cysteine residues while asterisk indicates conservation across all the selected accessions. BAD AVG GOOD

3.3.9 Phylogenetic analysis of hydrophobins

A phylogeny based on neighbour joining of the amino acid sequences was carried out to determine the relationship between *A. nidulans* hydrophobins and between *A. nidulans* and *A. niger* hydrophobins. Figure 3.8 shows the Treeview analysis of *A. nidulans* hydrophobins which was used to check the relationship between the class I hydrophobins and the unidentified class hydrophobins. The tree revealed the class I *A. nidulans* hydrophobins with accession number ANID_01837 and ANID_08803 to form the same clade (green rounded rectangle) with 100% bootstraps. The tree also showed class I ANID_08006 proteins (red rounded rectangle) forming a clade with the unidentified ANID_07539. Eighty five percent (85%) of the unidentified accessions (Figure 3.11) showed a distinct out-grouping from the identified class I hydrophobins.

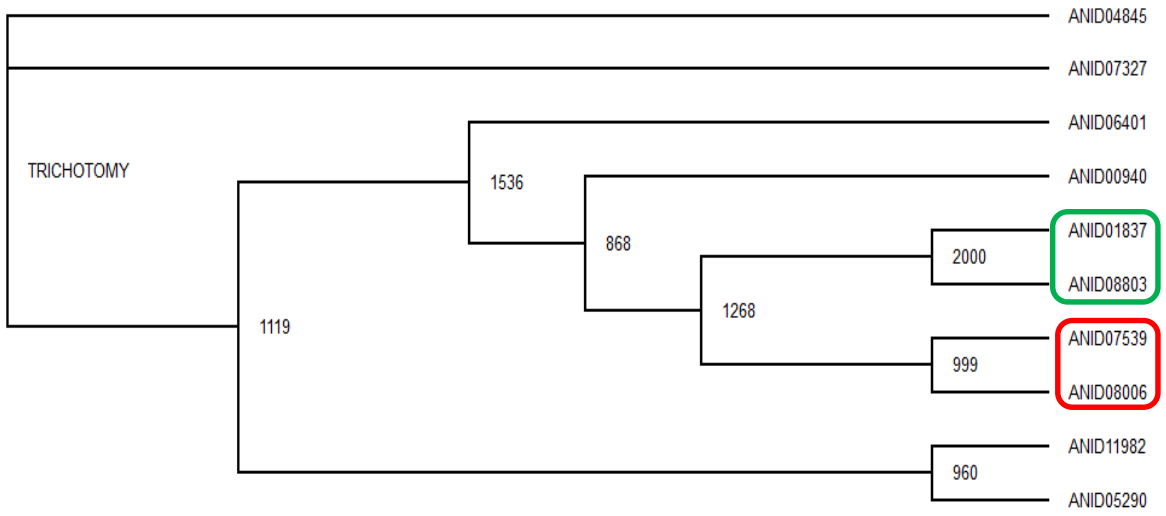


Figure 3.8: Treeview of *A. nidulans* hydrophobin protein sequences. Boxed accessions in red and green boxes highlight annotated hydrophobins belonging to class I while unboxed accessions are uncharacterised.

Figure 3:9 shows the Treeview analysis between *A. nidulans* and *A. niger* hydrophobin proteins. The result revealed *A. niger* (egw120216) hydrophobin proteins forming an out group with ANID_01837 and ANID_08803 which belong to the class I hydrophobin proteins (green rounded rectangle). The two *A. niger* hydrophobins An07g03340 and An08g09880 that were reported by Delmas *et al*., (2012) to be induced by the presence of lignocellulosic materials also formed an out group with the annotated gw15700 and trA5A8D1 hydrophobins respectively (red rounded rectangle) with a 100% bootstrap for both accessions.

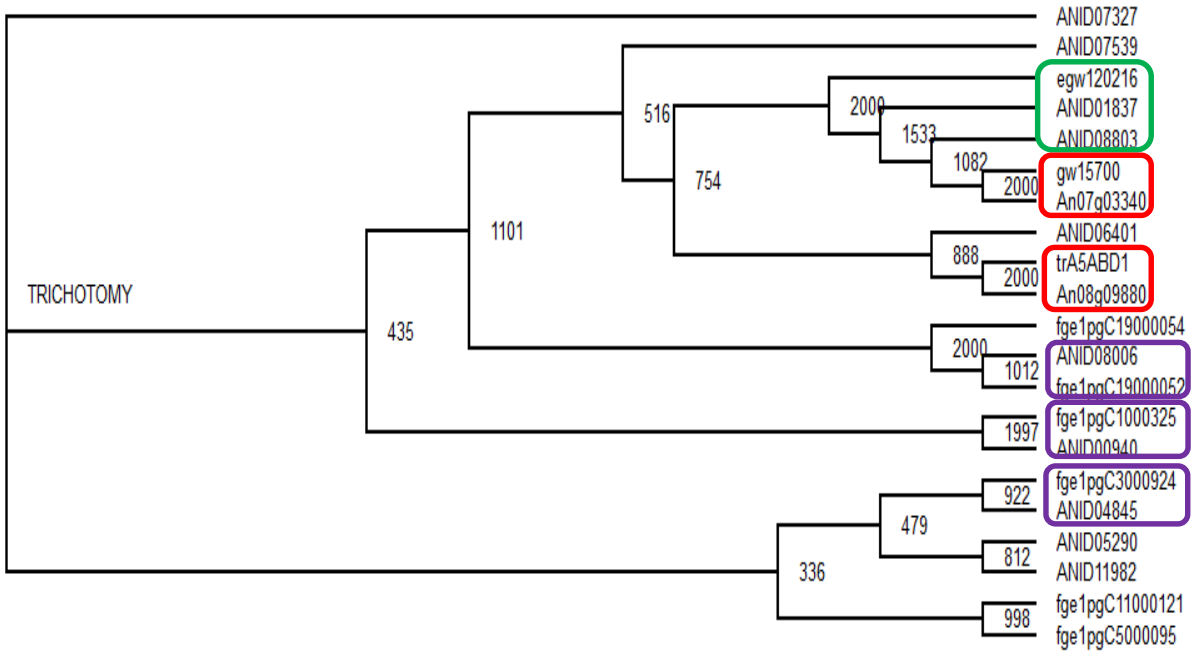


Figure 3.9: Treeview of *A. nidulans* hydrophobin protein sequences versus *A. niger* hydrophobin sequences. Green boxed accessions highlight annotated hydrophobins belonging to class I. Red boxes highlights *A. niger* hydrophobin reported by Delmas *et al*., (2012) that are induced by lignocellulose forming a clade with characterized hydrophobins (Littlejohn *et al*., 2012).

The Treeview result also showed some of the *A. nidulans* hydrophobin proteins forming a group with *A. niger* hydrophobin proteins (purple rounded rectangle). Some of the unclassified *A. nidulans* and *A. niger* hydrophobins were also found clustered together.

3.3.10 Selection of candidate β-glucosidases for expression analysis

Figure 3.10 is a summary of β-glucosidases screening based on the number of introns, annotation, length and similarity to attractive enzymes with multiple sequence alignments and phylogenetic trees used to define groups. The 7 selected sequences were further screened by comparing them to enzyme sequences from bacteria that are known to have attractive properties, such as acid and thermo-tolerance.

At the end of FASTA sequence screening, Afu6g12010 gene sequence was selected for artificial gene construction. Shorter genes had the attraction of being cheaper to synthesize whilst a reduction in predicted intron number may lessen the chances of intron annotation errors. The gene was artificially synthesized by GeneArt (Invitrogen, 2013). During the course of this work Lima *et al*., (2013) published a definitive description of the active site residues in β-glucosidases. TCoffee multiple sequence alignment of Afu6g12010 with AnBg11 (from Lima *et al*., 2013) revealed Afu6g12010 to lack active sites compared to the ten active sites in AnBg11 (Figure 3.11). This prompted further interrogation of the database in search of a sequence with the catalytic active sites. *N. fischeri* β-glucosidase sequence with accession number NFIA_027390 was finally selected and artificially synthesized by GeneArt (Invitrogen, 2013) because it possessed all the ten catalytic active sites (Figure 3.11).

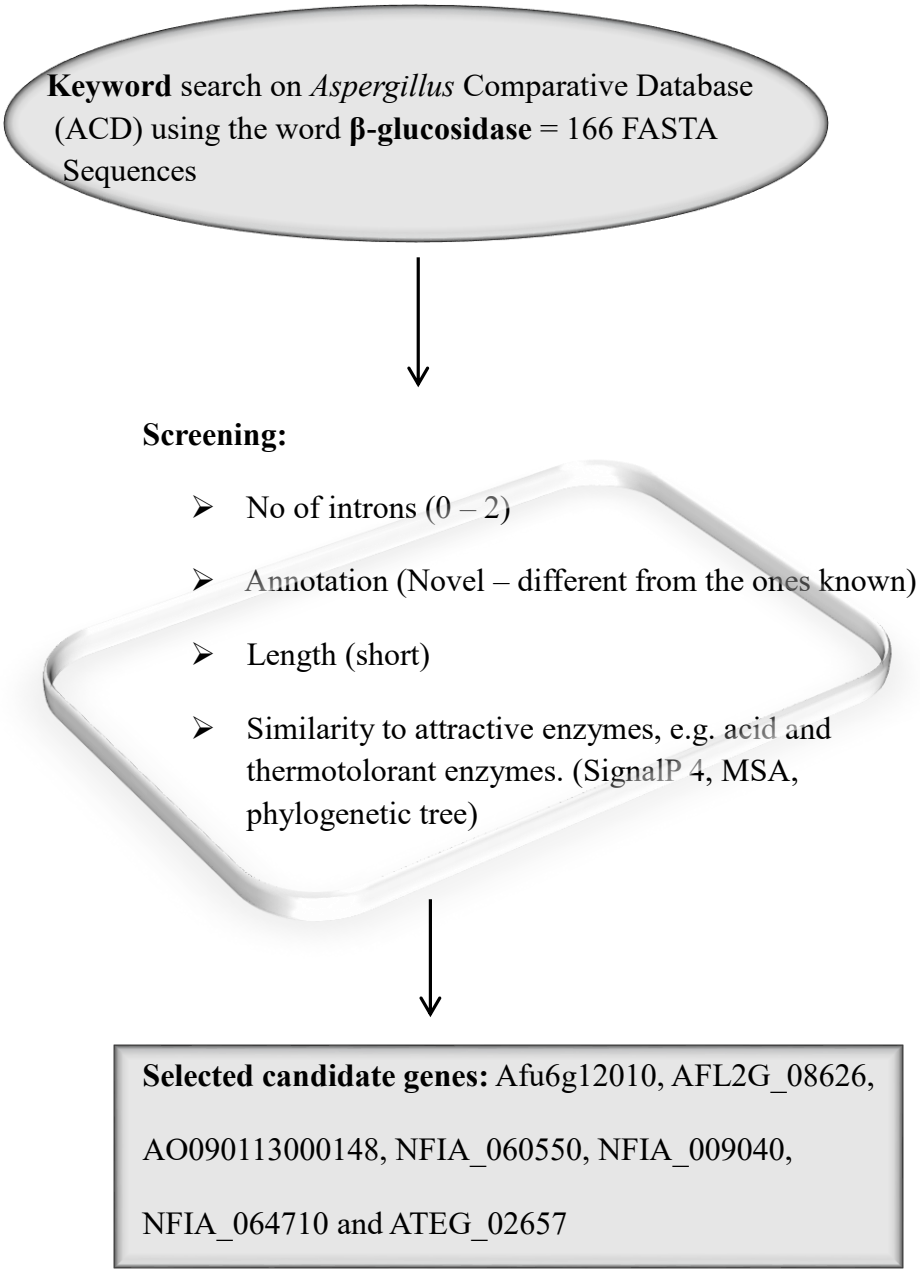


Figure 3.10: Flow diagram of screening to identify candidate β-glucosidase enzymes for artificial synthesis.

Figure 3.11 shows a complete sequence alignment of the select Afu6g12010 and NFIA_027390 with AnBg11 and KmBg11 (PDB code 3ABZ) which was carried out on the alignment program TCoffee (Notredame *et al*., 2000) after the removal of signal peptides to generate a multiple sequence alignment. The multiple sequence alignment results revealed poor alignment between the initially selected Afu6g12010 with AnBg11 and KmBg11 while there is a good alignment between NFIA_027390 with AnBg11 and KmBg11. Multiple sequence alignment also revealed accession NFIA_027390 to have all the ten core catalytic active sites while none is present in Afu6g12010.

The PA-14 domain is completely missing in Afu6g12010. Also, the N-terminal domain, which is suggested to act as a solubility enhancers for the folding C-terminal domains in vivo (Kim[1] *et al*., 2007), was completely missing in Afu6g12010, NFIA_027390 and KmBg11.

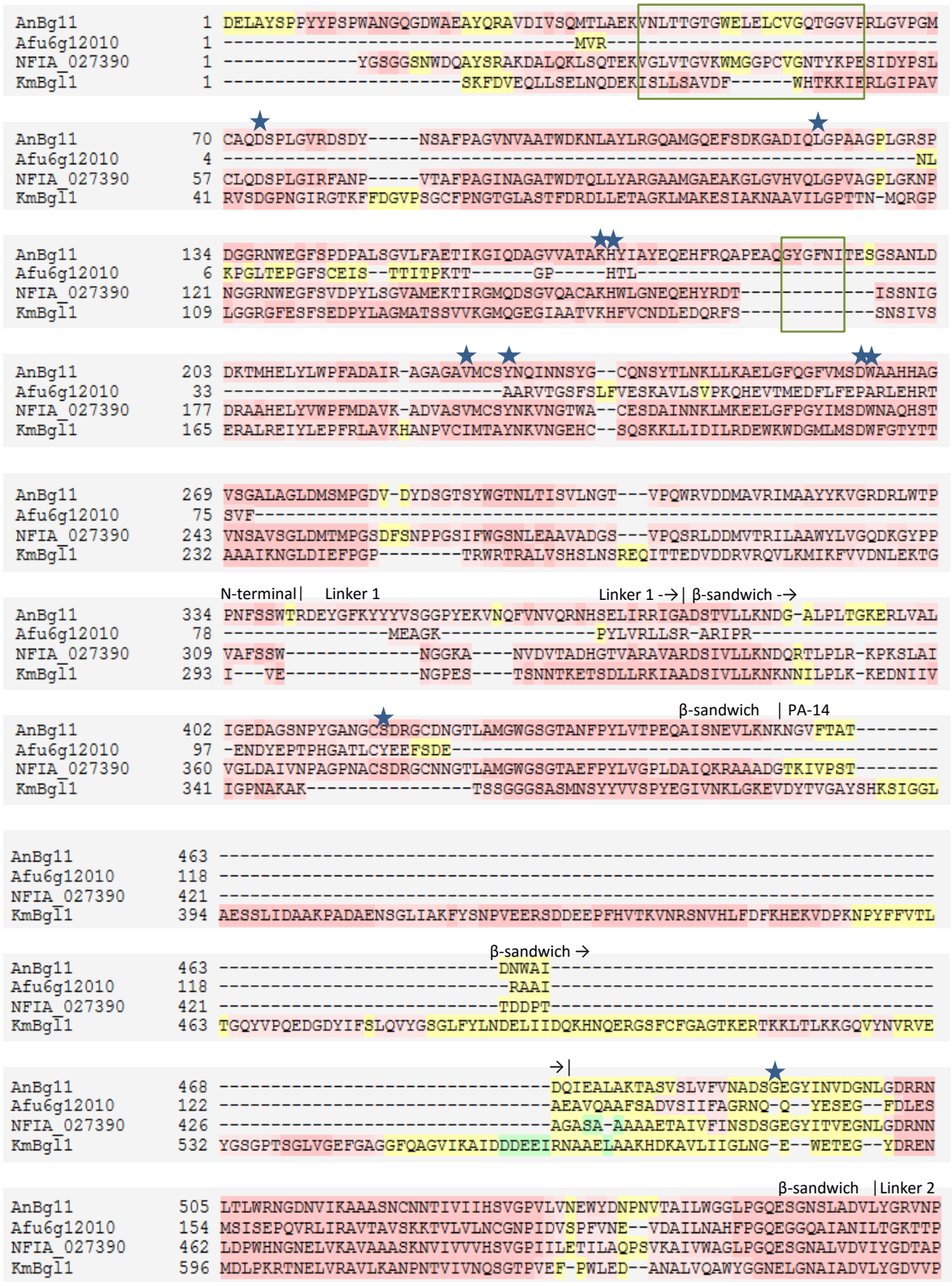

AnBg11 1 DELAYSPPYYPSPWANGQGDWAEAYQRAVDIVSQMTLAEKVNLTTGTGWELELCVGQTGGVPRLGVPGM
Afu6g12010 1 ----------MVR----------
NFIA_027390 1 ----------YGSGGSNWDQAYSRAKDALQKLSQTEKVGLVTGVKWMGGPCVGNTYKPESIDYPSL
KmBgl1 1 ----------SKFDVEQLLSELNQDEKISLLSAVDF------WHTKKIERLGIPAV
AnBg11 70 CAQDSPLGVRDSDY-----NSAFPAGVNVAATWDKNLAYLRGQAMGQEFSDKGADIQLGPAAGPLGRSP
Afu6g12010 4 ----------NL
NFIA_027390 57 CLQDSPLGIRFANP-----VTAFPAGINAGATWDTQLLYARGAAMGAEAKGLGVHVQLGPVAGPLGKNP
KmBgl1 41 RVSDGPNGIRGTKFFDGVPSGCFPNGTGLASTFDRDLLETAGKLMAKESIAKNAAVILGPTTN-MQRGP
AnBg11 134 DGGRNWEGFSPDPALSGVLFAETIKGIQDAGVVATAKHYIAYEQEHFRQAPEAQGYGFNITESGSANLD
Afu6g12010 6 KPGLTEPGFSCEIS--TTITPKTT------GP-----HTL----------
NFIA_027390 121 NGGRNWEGFSVDPYLSGVAMEKTIRGMQDSGVQACAKHWLGNEQEHYRDT----------ISSNIG
KmBgl1 109 LGGRGFESFSEDPYLAGMATSSVVKGMQGEGIAATVKHFVCNDLEDQRFS----------SNSIVS
AnBg11 203 DKTMHELYLWPFADAIR-AGAGAVMCSYNQINNSYG--CQNSYTLNKLLKAELGFQGFVMSDWAAHHAG
Afu6g12010 33 ----------AARVTGSFSLFVESKAVLSVPKQHEVTMEDFLFEPARLEHRT
NFIA_027390 177 DRAAHELYVWPFMDAVK-ADVASVMCSYNKVNGTWA--CESDAINNKLMKEELGFPGYIMSDWNAQHST
KmBgl1 165 ERALREIYLEPFRLAVKHANPVCIMTAYNKVNGEHC--SQSKKLLIDILRDEWKWDGMLMSDWFGTYTT
AnBg11 269 VSGALAGLDMSMPGDV-DYDSGTSYWGTNLTISVLNGT---VPQWRVDDMAVRIMAAYYKVGRDRLWTP
Afu6g12010 75 SVF----------
NFIA_027390 243 VNSAVSGLDMTMPGSDFSNPPGSIFWGSNLEAAVADGS---VPQSRLDDMVTRILAAWYLVGQDKGYPP
KmBgl1 232 AAAIKNGLDIEFPGP--------TRWRTRALVSHSLNSREQITTEDVDDRVRQVLKMIKFVVDNLEKTG
N-terminal| Linker 1
Linker 1 -→| β-sandwich -→
AnBg11 334 PNFSSWTRDEYGFKYYYVSGGPYEKVNQFVNVQRNHSELIRRIGADSTVLLKNDG-ALPLTGKERLVAL
Afu6g12010 78 ----------MEAGK----------PYLVRLLSR-ARIPR----------
NFIA_027390 309 VAFSSW----------NGGKA----NVDVTADHGTVARAVARDSIVLLKNDQRTLPLR-KPKSLAI
KmBgl1 293 I---VE----------NGPES----TSNNTKETSDLLRKIAADSIVLLKNKNNILPLK-KEDNIIV
β-sandwich | PA-14
AnBg11 402 IGEDAGSNPYGANGCSDRGCDNGTLAMGWGSGTANFPYLVTPEQAISNEVLKNKNGVFTAT--------
Afu6g12010 97 -ENDYEPTPHGATLCYEEFSDE----------
NFIA_027390 360 VGLDAIVNPAGPNACSDRGCNNGTLAMGWGSGTAEFPYLVGPLDAIQKRAAADGTKIVPST--------
KmBgl1 341 IGPNAKAK----------TSSGGGSASMNSYYVVSPYEGIVNKLGKEVDYTVGAYSHKSIGGL
AnBg11 463 ----------
Afu6g12010 118 ----------
NFIA_027390 421 ----------
KmBgl1 394 AESSLIDAAKPADAENSGLIAKFYSNPVEERSDDEEPFHVTKVNRSNVHLFDFKHEKVDPKNPYFFVTL
β-sandwich →
AnBg11 463 ----------DNWAI----------
Afu6g12010 118 ----------RAAI----------
NFIA_027390 421 ----------TDDPT----------
KmBgl1 463 TGQYVPQEDGDYIFSLQVYGSGLFYLNDELIIDQKHNQERGSFCFGAGTKERTKKLTLKKGQVYNVRVE
→|
AnBg11 468 ----------DQIEALAKTASVSLVFVNADSGEGYINVDGNLGDRRN
Afu6g12010 122 ----------AEAVQAAFSADVSIIFAGRNQ-Q--YESEG--FDLES
NFIA_027390 426 ----------AGASA-AAAAETAIVFINSDSGEGYITVEGNLGDRNN
KmBgl1 532 YGSGPTSGLVGEFGAGGFQAGVIKAIDDDEEIRNAAELAAKHDKAVLIIGLNG-E--WETEG--YDREN
β-sandwich |Linker 2
AnBg11 505 LTLWRNGDNVIKAAASNCNNTIVIIHSVGPVLVNEWYDNPNVTAILWGGLPGQESGNSLADVLYGRVNP
Afu6g12010 154 MSISEPQVRLIRAVTAVSKKTVLVLNCGNPIDVSPFVNE--VDAILNAHFPGQEGGQAIANILTGKTTP
NFIA_027390 462 LDPWHNGNELVKAVAAASKNVIVVVHSVGPIILETILAQPSVKAIVWAGLPGQESGNALVDVIYGDTAP
KmBgl1 596 MDLPKRTNELVRAVLKANPNTVIVNQSGTPVEF-PWLED--ANALVQAWYGGNELGNAIADVLYGDVVP

Figure 3.11: Multiple sequence alignment of β-glucosidases from *Aspergillus niger* (AnBg11), *Aspergillus nidulans* (Afu6g12010), *Neosartorya fischeri* (NFIA_027390) and *Kluyveromyces marxianus* (KmBg11.1). Symbols: Catalytic site (★); helix –A and –B (green box), interagent amino acids from linker 2 and N-terminal domain (black boxes) (Lima *et al*., 2013)

Figure 3.12 shows a phylogenetic tree (neighbour joining) of the selected accessions along with other fungal and bacterial FASTA sequences. In particular, Afu6g12010 is shown to form a group with bacterial sequences.

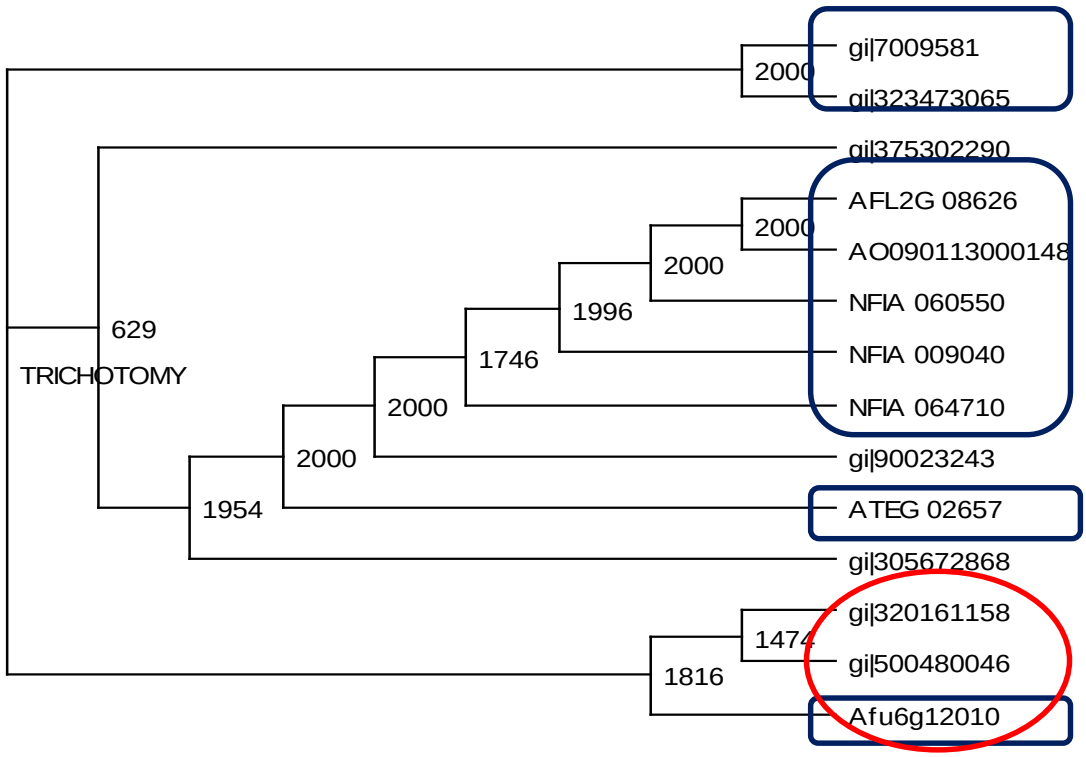


Figure 3.12: Treeview analysis of selected *Aspergillus* β-glucosidases compared with bacterial enzymes (without signal peptides - shown with values out of 2000 bootstraps for branch points). Rounded blue rectangle: Fungal species; Others: Bacterial species; Red oval: Bacteria and fungi forming the same clade. gi|7009581|: β-glucosidase *A. niger*; gi|323473065|: β-glucosidase from *A. fumigatus*; gi|375302290|: β-glucosidase from *Acetobacterium woodii*; AFL2G_08626: Hypothetical β-glucosidase from *A. flavus*; AO090113000148: Hypothetical β-glucosidase from *A. oryzae*; NFIA_060550, NFIA_009040 and NFIA_064710: Hypothetical β-glucosidase from *N. fischeri*; gi|90023243|: putative β-glycosidase *Saccharophagus degradans*; ATEG_02657: Hypothetical β-glucosidase from *A. terreus*; gi|305672868|: β-hexosaminidase from *Bacillus subtilis*; ; gi|320161158|: β-glucosidase from *Anaerolinea thermophila* ; gi|500480046|: β-glucosidase from *Thermotoga petrophila*; Afu6g12010: β-glucosidase from *A. fumigatus*.

3.4 Discussion

3.4.1 Manual annotation of β-glucosidases in *Aspergillus*

β-glucosidases work in harmony with exoglucanase and endoglucanase to hydrolyse cellulose to fermentable sugars (Wang *et al*., 2011; Liu *et al* ., 1996). The protein sequences of potential β-glucosidases from the *Aspergillus* genus in this study were manually searched and classified for family name. Literature surveys (Henrissat, 1991; Sørensen *et al*., 2013) showed that two methods are generally used in the classification of β-glucosidases which are based on substrate specificity and nucleotide sequence identity. β-glucosidases from bacteria, fungi, plant and mammalian origin belong to GH1 and usually possess a significant level of galactosidase activity in addition to β-glucosidase activity while β-glucosidases of fungi, bacteria and plants are classified into GH3. From a structural point of view, the GH1 family are said to catalyse substrates following the β-retaining action mechanism that employs glutamic acid as a catalytic nucleophile, while GH3 utilize an aspartic acid residue in their nucleophile attack of the substrate (Lima *et al*., 2013). The GH1 β-glucosidases consist of proteins with $(\beta/\alpha)_8$-barrel structures in contrast to the active site of GH3 enzyme which comprises of two domains (Cairns and Esen, 2010). CAZy classification of GH has placed β-glucosidases in GH family 1 and 3 based on their amino acid sequence structure (Coutinho *et al*., 2009).

This chapter describes the manual annotation of β-glucosidases in comparison with the report of Coutinho *et al*., (2009). It should be noted from Table 3.2 that 29 of the β-glucosidases of the *Aspergillus* genus belong to GH1 family while 137 belong to the GH3 family across all the species. The Table also shows that 3 of the GH1 proteins and 20 of GH3 proteins are from *A. nidulans*. The number in the GH1 family identified from *A. nidulans* in this research is in agreement with the report of Coutinho *et al*., (2009) who identified 3 GH1 family β-

glucosidases from *A. nidulans*, except that two of the GH1 proteins (AN10127 and AN10353) reported by Coutinho *et al*., (2009) are missing in this study while ANID_10375 and ANID_10124 reported in this study are missing in the Coutinho *et al*., (2009) report. There may be several reasons for the difference, for example, the sequences may be the same but deposited in the database with different accession identification numbers. Updating the database since 2009 may change some annotations. Meanwhile, there is disagreement in the number of GH3 proteins in *A. nidulans*. This work reported 20 GH3 proteins while Coutinho *et al*., (2009) reported 21 GH3 proteins from *A. nidulans*. The disagreement could be as a result of misannotation of one or more sequences. The differences in the number of GH1 and GH3 in various *Aspergillus* species (Table 3.2) could be partly as a result of the differences in the genome sizes of the species or lifestyles (Galagan *et al*., 2005). There was agreement in the number of *A. oryzae* β-glucosidase FASTA sequences reported in this study and the report of Coutinho *et al*., (2009) except for the accession AO090005000706 reported by Coutinho *et al*., (2009) as a β-glucosidase belonging to GH1 family. In this study, AO090005000706 was identified as a serine-tRNA ligase and not β-glucosidase.

In contrast to the 20 *A. niger* β-glucosidases identified by Coutinho *et al*., (2009), the combined keyword/Blast approach in this study identified 14. It was difficult establishing the β-glucosidase FASTA sequences that are missing in both reports because the sequence identification numbers or accession numbers reported in the two reports are different. This also could be ascribed to misannotation. Due to the rapid release of new data from genome sequencing projects, the majority of protein sequences in public databases have not been experimentally characterized; rather sequences are annotated using computational analysis, thereby increasing the level of misannotation (Schnoes *et al*., 2009). A specific example from this study highlights the use of information from the *Aspergillus* comparative database,

Uniprot and FungalDB to provide annotations. This may be a problem because the original source information from the databases may not actually refer to a specific functional annotation but rather may only refer to a group of proteins sharing the same structural domain but representing multiple different functions (Schnoes *et al*., 2009).

3.4.2 Multiple sequence alignments and phylogenetic analysis of β-glucosidases

Initial annotations of Afu6g12010 with multiple sequence alignments and phylogenetic tree analysis (Figure 3.3 and 3.4) predicted similarity to attractive enzymes (e.g. thermo-tolerance) from the NCBI and CAZy databases. A number of the potential β-glucosidases lacked some of the conventional active site residues, for example, the Afu6g12010 gene had no introns, and its product had a small size (409 amino acids) and was predicted to belong to the GH3 family. Additional multiple sequence alignment studies on Afu6g12010 with protein sequences from AnBg11 and AaBg11 (Lima *et al*., 2013) revealed that Afu6g12010 lacked most of the essential ten β-glucosidase active sites, leading to the question of whether the encoded Afu6g12010 protein is active in cellulose hydrolysis. Most interestingly, multiple sequence alignments also predicted an unexpected similarity of fungal proteins to those of enzymes from bacteria, suggesting possible horizontal gene transfer events from bacteria. For example, Figure 3.4 highlights the unexpected association in phylogenetic trees of *Aspergillus* enzymes with bacterial counterparts. The presence of the acquired genes could be associated with evolutionary adaptations for nutrition to allow the colonization of new environments (Hakkinen *et al*., 2012). A further example could be seen in Figure 3.2 where some fungal amino acid insertions (Lima *et al*., 2013) are missing towards the C-terminal in AN1804.2 suggesting that they may be of bacterial origin and not of fungal. Another example is seen in Figure 3.4 where AN1804.2 and two other *Aspergillus* enzymes grouped with a *B. subtilis* enzyme.

Fungal genes are known to usually contain introns while genes from eubacteria do not have introns. *Aspergillus* sequences such as those from AO090005000337 and AN1804.2 do not have introns while AN1551.2 has only one intron (Table 3.3). The phylogenetic classification (Figure 3.4) of β-glucosidase sequences from AO090005000337, AN1804.2 and AN1551.2 into the same group with *B. subtilis,* which is a bacterial species, is a possible support of the idea that the encoding genes arose from horizontal gene transfer.

Phylogenetic tree analysis was carried out to determine evolutionary relationships between the β-glucosidase proteins. A list of β-glucosidases with basic information about number of introns and signal peptide of detailed annotated genes to highlight individual accessions with/without introns is shown in Appendix 28. Coutinho *et al*., (2009) stated that 28.4% of *A. nidulans* open reading frames (ORF's) did not have secretional signal peptides. The results from this current search revealed that 46.67% of the *A. nidulans* ORF's have secretion signal peptides. Horizontal gene transfer (HGT) in microbes has played an important role in their evolution and in the generation of genes involved in the synthesis of cellulase enzymes which degrade cellulose. The process and mechanisms involved in the exchange of DNA between distant species is unknown (Mallet *et al*., 2010) but horizontal gene transfer between bacteria and fungi in nature is well documented (Schmitt and Lumbsch, 2009). Hakkinen *et al.*, (2012) recently showed the horizontal gene transfer of an endoglucanase gene from bacteria to fungi (from *Bacillus subtilis* to *Trichoderma*). Another classic example of HGT is documented by Ubhayasekera and Karlsson (2012), where *Streptomyces* genes are shown to have orthologues in *Hypocrea* (*Trichoderma*).

The Phylogenetic trees in Figure 3.4 showed an unexpected association between fungal and bacterial proteins. Trees show bacterial enzymes unexpectedly not rooting the trees but associated with some *Aspergillus* candidates. For instance, the phylogenetic distribution shows

Afu6g12010 (Figure 3.4) which is distantly grouped from related fungal species but closely grouped with a bacterial protein from *Gluconacetobacter xylinus*. The lack of grouping of Afu6g12010 with proteins from related species could alternatively be as a result of convergent evolution where proteins have evolved from different species to fill the same role or potential horizontal gene transfer. Another example from the tree analysis is where *Thermotoga petrophila* and *Bacillus subtilis*, both of which are bacterial species, are respectively grouped with examples from fungal species. A possible explanation to why fungal species retain bacterial genes could be because of lifestyle, where the organism kept the gene for adaptation to allow the colonization of new environments and nutritional purposes. This aspect is explored further in Chapter 4.

In a study on phylogenetic distribution of cellulases in bacteria, Berlemont and Martiny (2013) reported that many bacteria (termed opportunistic bacterial strains) produce only β-glucosidases while a small number of potential bacteria cellulose degraders produce both other cellulases and β-glucosidases. Many bacteria therefore act as opportunists, relying on other species with a complete repertoire of cellulose degrading enzymes to provide cellobiose. In contrast *Aspergillus* species seem to be self-sufficient for cellulose degradation, encoding the full range of enzymes required (Coutinho *et al*., 2009). However, the present work suggests that *Aspergillus* may have acquired specific β-glucosidase genes from bacteria. It is striking that all the *Aspergillus* species studied encode many β-glucosidases, suggesting that they may have specific or non-redundant roles.

A hydrophobicity plot is a quantitative analysis of the degree of hydrophobicity of amino acid residues and is used to identify or characterize possible structure of protein. Figure 3.5 depicts an example of the hydrophobicity of β-glucosidases from *A. niger* (AnBg11) and *Gluconacetobacter xylinus* (*G. xylinus*). The result shows similarity throughout the β-

glucosidase proteins and shows the proteins to be hydrophilic in both fungal and bacterial β-glucosidases. Enzymes as proteins are soluble in water and have advantages for the treatment of substrates which are insoluble such as cellulose. The hydrophilic and solubility nature of β-glucosidase offer some unique advantages such as ease of dispersal during the hydrolysis process, and also precipitation during the purification process (Onyike *et al*., 2008). Hence, a bioinformatics approach can identify candidate enzymes and make initial predictions as to some likely biochemical properties. It is also a useful tool for predicting the secondary structures of protein sequences using programs such as PSIPRED (Figure 3.6). Secondary and tertiary profiles could be used to resolve whether some of the hypothetical proteins sequences really do represent β-glucosidases, for e.g. those with few active sites.

3.4.3 Multiple sequence alignments and phylogenetic analysis of hydrophobins

Hydrophobins are small secreted proteins containing a characteristic pattern of eight cysteine residues which are divided into two classes based on their hydropathy patterns and physical properties (Jensen *et al*., 2010). Multiple sequence alignment analysis of the ten *A. nidulans* hydrophobin proteins carried out using TCoffee (Notredame *et al*., 2000) analysis of the proteins revealed three of the proteins which have been previously described (Littlejohn *et al*., 2012) to fit into the class I cysteine model. Seven of the proteins neither belong to class I nor class II hydrophobin class model placing them as an intermediate form. These results and analysis are in agreement with the report of Littlejohn *et al*., (2012) where ANID_01837, ANID_08006 and ANID_08803 were classified as belonging to an intermediate novel class of hydrophobins based on the Kershaw and Talbot (1998) sequence system model.

Comparison studies of hydrophobins identified by Linder *et al*., 2005 were carried out by drawing trees using sequences starting from the first cysteine residue because sequences

preceding the first cysteine differ in length and composition (Wösten, 2001). The alignment in this work was carried out by removal of the signal peptide only, this is because it has been noted that there is uncertainty in the production of any multiple alignment and the choice of starting amino acid residue can also affect subsequent tree structure (Littlejohn *et al*., 2012).

The association between *A. nidulans* hydrophobins and the relationship between *A. nidulans* and *A. niger* hydrophobin proteins were analyzed using ClustalX (Larkin *et al*., 2007). Comparison of the ten *A. nidulans* hydrophobin proteins (Figure 3.8) shows a clear separation between the annotated class I hydrophobins and the uncharacterized hydrophobins which may infer that there is divergent evolution between the proteins. Treeview analysis comparing *A. nidulans* with *A. niger* hydrophobin proteins (Figure 3.9) showed association between the hydrophobin proteins from the two different species. For example, ANID_08006, ANID_00940 and ANID_04845 have clustered closely with fge1pgC19000052, fge1pgC1000325 and fge1pgC3000924 respectively. This relationship between the two groups of proteins may simply reflect common ancestry. Delmas *et al*., (2012) have reported two *A. niger* hydrophobins (An07g03340 and An08g09880) that are induced by the presence of lignocellulosic materials such as wheat straw, suggesting that these proteins may possess novel characteristics for lignocellulose degradation in biofuel production. The two hydrophobin proteins had signal peptides which were manually removed, suggesting the possibility of the proteins being secreted. These proteins were also clearly shown (Figure 3.9) to closely cluster together with gw15700 and trA5ABD1 respectively with a bootstrap each of 100%. The nearest *A. nidulans* hydrophobin to An08g09880 is ANID_06401 suggesting that this may be a more likely candidate to be lignocellulose induced.

3.4.5 Conclusion

In this study, the manual annotations, multiple sequence alignments and phylogenetic analysis of β-glucosidases on the *Aspergillus* comparative database, UNIPROT and FungiDB have provided updated annotations of the β-glucosidases. A combined keyword search/Blast approach to identify β-glucosidases underestimated the numbers of *A. niger* β-glucosidases probably because of misannotation, database updates and/or differences in accession numbers. Evidence for HGT bacterial origin of some β-glucosidases in *A. nidulans*, for example AN1804.2, was provided by their lack of introns, absence of some fungal specific amino acid insertions in their sequence, automatic annotation as "periplasmic" and unusual positions in phylogenetic trees showing similarities to bacterial proteins. Multiple sequence alignment and phylogenetic trees of hydrophobins from *A. nidulans* and *A. niger* showed distinct clusters of proteins with likely similar functions.

Chapter 4

Screening of fungal and bacterial strains for cellulase activity, purification and characterization of recombinant β-glucosidases

4.1 Introduction

In the biofuel industry, cellulases are the key enzymes used in the utilization of biomass (renewable energy) by conversion of abundant cellulose resource into fermentable sugars. The breakdown of lignocellulosic waste by microbes is usually accomplished by the combined effort of several enzymes, the most outstanding of which are the cellulases produced by a number of microorganisms (Sukumaran *et al*., 2005). Cellulase-producing microbes find application in biofuel industries and constitute a major group of industrial enzymes. Cellulolytic microbes generally do not use lipids or proteins as source of energy for growth but primarily degrade carbohydrates and use its product as a source of energy (Lynd *et al*., 2002).

Enzyme yield normally depends on factors like temperature, pH, carbon sources and incubation periods (Gautam *et al*., 2011). The pH and temperature of a solution are important factors that influence enzyme production and activity. For a successful fermentation process, investigation is required to establish the optimum conditions for enzyme production. The enzymatic hydrolysis of cellulose by cellulolytic microorganisms has been documented as an attainable substitute for the conversion of lignocellulosic material into biofuel (Lynd *et al*., 2005; Lowe *et al*., 1987). Although fungi are the main cellulase enzyme-producing microorganisms, bacteria also produce significant cellulase activity (Penttilà *et al*., 1986; Tomme *et al*., 1988).

Fungal and bacterial strains possessing cellulose degrading enzymes have been isolated and screened for many years (Ram *et al*., 2014). Examples of some fungi that have been studied for β-glucosidase production include *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus saccharolyticus*, *Trichoderma koningii*, *Trichoderma reesei*, *Neosartorya sicheri* and *Fusarium solani* (Sørensen *et al*., 2013). *Trichoderma reesei* has strong cellulose degrading properties and its cellulase systems have been widely studied (Tiwari *et al*., 2013). A number

of bacteria such as *Bacillus subtilis* (Bagudo *et al*., 2014) and some *Clostridium* species (Menedez *et al*., 2015) have also been reported for their cellulolytic activities. Previous research has shown that the cost of enzyme production is associated with the specific cellulase producing microbial strains (Chahal *et al*., 1992; Duff and Murray, 1996; Reczey *et al*., 1996; Omojasola and Jilani, 2008).

Screening of cellulase producing microorganisms can be carried out qualitatively on agar plates using CMC as a carbon source for microbial growth with indicators such as Congo red (Sazci *et al*., 1986; Florencio *et al*., 2012) or quantitatively *via* biochemical assay using spectrophotometric techniques and substrates such as p-nitrophenyl- β-Dglucopyranoside (pNPG) (Ghose and Bisaria, 1987) and 3,5-dinitrosalicyclic acid (DNS) (Cianchetta *et al*., 2010; Onyike *et al*., 2008). The qualitative screening method is a simple, rapid and cheap assay method as compared to quantitative methods, which, whilst precise, are slow and requires expensive materials and equipment (e.g. Onyike *et al*., 2008). Potential cellulase/β-glucosidase producing microorganisms used in these studies are described in Table 2.1 and Table 2.2. A rapid, plate-based method of screening for cellulase activity is required for fermentation studies, when large numbers of samples need to be quickly assayed to identify optimum production conditions. The microorganisms were selected because of their availability in the University of Wolverhampton culture collection centre and also based on a literature search.

Bioinformatics tools were used in chapter 3 to interrogate databases for screening and characterization of β-glucosidases with novel properties for direct artificial gene synthesis and cloning. In contrast, Chapter 4 involves the direct screening of microorganisms on agar plates focused on a qualitative evaluation of strains to produce cellulolytic enzymes.

Bauer *et al*., (2006) have already reported the successful cloning and expression of a number of plant and fungal cellulases using a *Pichia pastoris* expression. Bauer *et al*., (2006) reported the production of five putative β-glucosidase enzymes from *A. nidulans* with the accession numbers AN2227.2, AN2612.2, AN0712.2, AN1551.2 and AN1804.2. The recombinant *Pichia* strains of these accessions carrying the β-glucosidase genes from *A. nidulans* are deposited in the Fungal Genetic Stock Centre (FGSC) USA. The clones were obtained and exploited for β-glucosidase production.

Figure 4.1 and Figure 4.2 below summarise the features of the pPICZ A, B and C; and pPICZα A, B and C vectors as examples of expression vectors*.

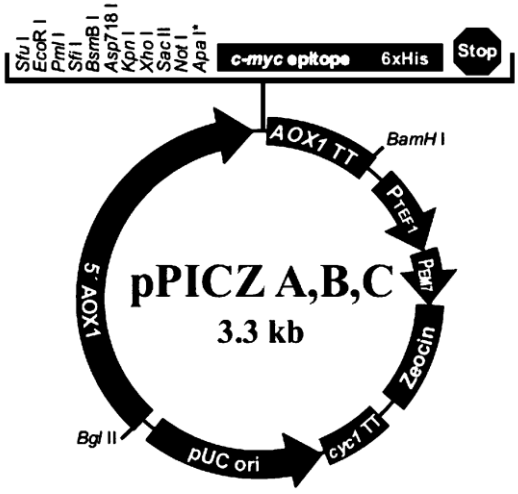


Figure 4.1: Expression vector diagram of pPICZ (Invitrogen, 2010)

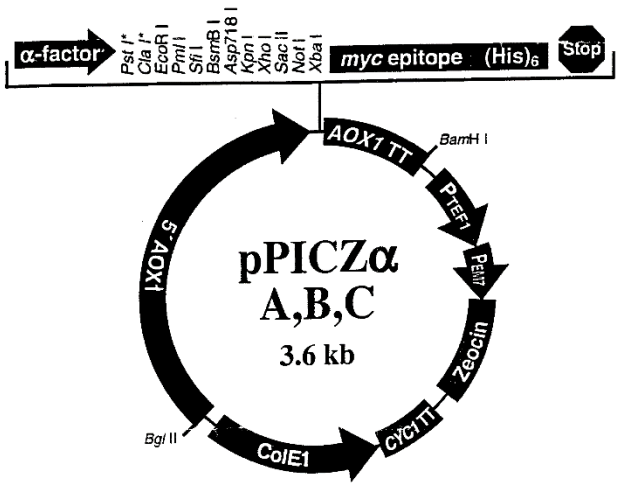


Figure 4.2: Expression vector diagram of pPICZα (Invitrogen, 2010)

Both vectors contain the 5' *AOX1* promoter that allows methanol-inducible, high-level expression in *Pichia* and targets plasmid integration to the *AOX1* locus. The series encode a pPICZα native alpha signal secretion factor that allows for efficient secretion of most proteins from *Pichia*. The expression vectors have multiple cloning sites with 10 unique restriction sites that allow insertion of a foreign gene into the expression vector (EasySelect^{TM} Pichia Expression Kit user guide, 2010). In shake flasks fermentation using *Pichia pastoris*, commonly one or two methanol pulses are added per day for induction. Such a feeding procedure leads to carbon starvation phases, which may enhance proteolytic activities and therefore cause product losses (Panula *et al*., 2014).The *Pichia* expression system is discussed in Chapter 1, section 1.2.10.

Protein purification involves the concentration of the protein, separation of the different proteins using chromatographic techniques and performing SDS-PAGE analysis to verify the presence of the protein (Ward and Swiatek, 2009). Several researchers have successfully

produced and purified hydrophobins and β-glucosidases from fungal and bacterial sources using *Pichia pastoris* (Ng *et al*., 2000; Askolin *et al*., 2001; Niu *et al*., 2012; Kaur *et al*., 2007; Meko'o *et al*., 2012; Bauer *et al*., 2006; Chen *et al*., 2011; Chang *et al*., 2012; Sørensen *et al*., 2013). The use of the *Pichia pastoris* system in the heterologous expression of hydrophobin is well documented (Niu *et al*., 2012; Stubner *et al*., 2010; Wang *et al*., 2010; Kottmeier *et al*., 2012). Niu *et al*., (2012) expressed a 7.5 kDa recombinant hydrophobin HFBI (rHFBI) from *Trichoderma reesei* and it was shown that rHFBI was a strong gushing inducer in beer. Stubner *et al*., (2010) also expressed a class II hydrophobin FcHyd5p from *Fusarium culmorum* and demonstrated that FcHyd5p can induce spontaneous over-foaming of carbonated liquids. Similarly, Chen *et al*., (2011) expressed a 76 kDa β-glucosidase from *Trichoderma reesei* using *Pichia pastoris*. Under the control of methanol-inducible alcohol oxidase (AOX) promoter and using *Saccharomyces cerevisiae* secretory signal peptide (α-factor), the recombinant β-glucosidase was expressed and secreted into the culture medium. A heterokaryon 28, derived through protoplast fusion between *Aspergillus nidulans* and *Aspergillus tubingensis* (Dal8), was subjected to cyclic mutagenesis by Kaur *et al*., (2014) followed by selection on increasing levels of 2-deoxy glucose (2-DG) as a selection marker. The derived deregulated cellulase hyper-producing mutant 64, when compared to fusant 28, produced 7.8 folds β-glucosidase under shake cultures.

The aim of this chapter is to:

i. Establish conditions for the production of β-glucosidase from native bacterial and fungal strains as well as *Pichia* clones expressing cloned *A. nidulans* β-glucosidases.
ii. Characterize pH and temperature profiles of two β-glucosidases expressed in *P. pastoris*.

4.2 Materials and methods

4.2.1 Source of microorganisms

The fungal and bacterial microorganisms used in this study are listed in Table 2.2 and Table 2.3. The screening of activity on CMC plates in all the subsequent sections were based on crude cellulase enzyme extract from the organisms. The five *Pichia* clones carrying *A. nidulans* in pPICZ vectors used are listed in Table 2.3.

4.2.2 Screening of Microorganisms

A total of 21 fungal (Table 2.1) and 8 bacterial (Table 2.2) strains were screened for cellulase activity. The screening method in this study was based on the assay method of Sazci *et al*., (1986), staining incubated agar plates with Congo red and distaining with NaCl as described in section 2.5. Cellulase activities were detected from the appearance of zones by substrate clearance and discoloration around the colonies (Figure 4.3 A and 4.3 B). The NaCl solution used for de-staining elutes the dye in the clearing zone where the cellulose has been degraded into simple sugar by the enzymatic activity. The screening strategy was performed based on pH, temperature and carbon source.

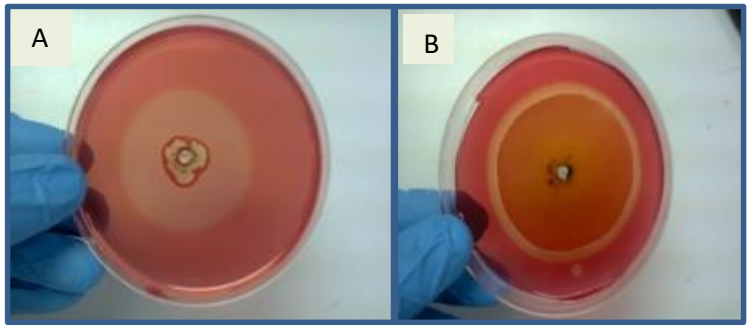


Figure 4.3: Example of zone of hydrolysis after incubation. A: crude cellulase of *A. niger* F212 at 45 ^{o}C without cells; B: *A. niger* F321 cells at 40 ^{o}C producing cellulase

4.2.3 Effect of pH on fungal and bacterial cellulase activity

To determine optimal pH of fungal and bacterial cellulase activity, experiments were carried out by incorporating CMC as carbon source into agar plates. Minimal medium (De-Vries *et al*., 2004) was used with supplementation of CMC at 5% w/v level. The pH of the medium was adjusted to pH 4, 5, 6, 7, 8 or 9 respectively. Into the appropriate wells on the agar plates, 40 µl of fungal and bacterial cellulase were placed as described in Chapter 2, section 2.3. Incubation for fungal cellulase was carried out for 1 day at 35^{o}C while that of bacteria was carried out for 3 days at 35^{o}C. Assays were conducted to determine enzyme activity from the appearance of zones by substrate clearance using the Congo red method.

4.2.4 Effect of temperature on fungal cellulase activity

The effect of temperature on crude fungal cellulase enzyme was studied using the Congo red clearing zone assay method. All organisms (Chapter2, Table 2.1) were initially grown on Malt Extract Agar (MEA) plates. Spores were aseptically picked from MEA plates and inoculated into 5 ml sterile nutrient broth in a McCartney bottle. The bottles were incubated at 30^{o}C in an orbital incubator shaker (Model G25, S/No 390534557 U/K) at 150 RPM for 2 days. After the incubation period, the samples were centrifuged at 3500 rpm (MSE MISTRAL 1000 centrifuge S/No M1000 240-V) to remove mycelia. The supernatant (crude enzyme) culture filtrate was filter sterilized into sterile Bijoux bottles using a sterile MILLEX^{o} GP Syringe Filter Unit (0.22 µm). Into the appropriate wells on the CMC agar plates, 40 µl of the crude enzyme (sample) or nutrient broth (Control) was placed and incubated for 2 days. Assays were carried out as outlined in Chapter 2, section 2.3 to determine the semi-quantitative cellulase activity.

4.2.5 Effect of carbon sources on fungal growth

The effect of carbon sources on fungal growth was studied by supplementing different carbon sources into agar plates. Minimal medium (De-Vries *et al*., 2004) was used with supplementation of Glucose, Cellobiose and CMC at 5% w/v level. For cellulose, Whatman filter paper (Cat No. 1001042) was used. The minimal medium used for the experiment had the following composition in g/l: $NaNO_3$ – 1, K_2HPO_4 – 1, KCl – 1, $MgSO_4$ – 0.5, Agar no. 2 – 15 and trace elements – 1ml. The medium was adjusted to pH 6.0 with 0.5 M NaOH and sterilized at 121^oC for 15 min. To 998 ml of the minimal medium, 2 ml vitamin solution was added using a sterile MILLEX^o GP Syringe Filter Unit (0.22 µm). Five grams of the substrate (carbon source) was weighed into sterilized flasks and 1ml of ether was added. After 15 min, the cotton plug was set loose to allow evaporation of ethanol. Autoclaved agar was added to the ether sterilized carbon source, swirled and then poured into plates. Sterilized agar plates without carbon source were used as a control. Filter paper used in this assay was placed on sterilized agar plates with no other carbon source. Spores were aseptically picked and inoculated into agar plates by point inoculation. Incubation was carried out at 35^oC for 2 days except for cellulose plates which were incubated for 6 days. Three diameters were measured per colony. After incubation periods, plate assays were carried out using the Congo red assay procedure described in section 2.5.

4.2.6 Small Scale Shake Flask Fermentation of *Pichia* strains containing AN2612.2, AN0712.2, AN1551.2, AN2227.2 and AN1804.2 (Bauer *et al*., 2006)

Buffered Methanol Complex Medium (BMMY - Yeast extract 1%, Peptone 2%, 100 mM potassium phosphate (pH 6.0), Yeast Nitrogen Base (YNB) 1.34%, Biotin 4 x 10^{-5} %, Methanol 0.5%) were used for the broth. 100 ml of BMMY was aseptically inoculated with *Pichia* strains containing either AN2612.2, AN0712.2, AN1551.2, AN2227.2 or AN1804.2.

Prepared inocula were incubated in an orbital shaker (150 rpm) at 30ºC for 48 hours. β-glucosidase enzymes were withdrawn from the culture broth after 0, 3, 6, 9, 12, 15, 18, 24, 48, 72 and 96 hours and were filter sterilized using a syringe filter (0.22 µm) to separate mycelial cells from culture filtrates. Crude enzyme samples (40 µl) were inoculated in duplicates on CMC supplemented agar plates and incubated at 37ºC for 48 hours. Assays were conducted to determine enzyme activity from the appearance of zones by substrate clearance using the Congo red method as described in section 2.3.

Growth was also monitored after 0, 3, 6, 9, 12, 15, 18, 24, 48, 72 and 96 hours on broth cultures. The cultures were diluted approximately from 10^{-1} to 10^{-8} using the Ringer's solution ¼ strength (1 tablet, made up of NaCl, KCl, CaCl and $NaHCO_3$ was completely dissolved in 500 ml deionized water and sterilized at 121ºC for 15 min). The serially diluted samples were inoculated in triplicate on MEA plates and viable cell count was carried out using the Miles and Misra method as described in section 2.5. All results were statistically analysed with GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

4.2.7 Small scale cultivation of the recombinant *P. pastoris* β-glucosidase clones

The experiment was carried out to determine protein production yield and activity of AN2227.2 and AN1804.2 (Chapter 3, Figure 3.2) which are reported by Bauer *et al*., (2006) as putative β-glucosidase enzymes from *A. nidulans* and are *Pichia* clones using pPICZ expression system (details in Chapter 2, Table 2.3). The engineered AN2227.2 or AN1804.2 was inoculated into 25 ml of Buffered Minimal Glycerol Medium (BMGY) medium in a 250 ml flask. The cell cultures were incubated on an orbital shaker at 30 ºC and 250 rpm for 16 - 18 hours to grow in the repressive medium before induction. When the culture concentration

reached an OD_{600} = 3, the cells were harvested by centrifuging at room temperature and 5000 rpm for 5 minutes. The cell pellet was re-suspended in 90 ml Buffered Methanol Complex Medium (BMMY - Yeast extract 1%, Peptone 2%, 100 mM potassium phosphate (pH 6.0), YNB 1.34%, Biotin 4 x 10^{-5}%, Methanol 0.5%) medium until OD_{6oo} = 1 to induce expression. The culture was incubated in an orbital shaker at 150 rpm and 30 ^{o}C for 4 days. The growth and induction took place in 1 litre flasks and the cell culture was induced every 24 hour after the start of cultivation with methanol to 0.5% of the total volume of the culture to compensate for methanol loss caused by evaporation from medium and uptake by cells.

Crude enzyme samples (1.5 ml) were withdrawn from the culture broth after 0, 6, 12, 24, 48, 72 and 96 hours and filter sterilized using syringe filter (0.22 µm) to separate mycelial cells from culture filtrates. Assays were conducted to determine enzyme activity using p-nitrophenyl- β-D-glucopyranoside (pNPG) as substrate as described in section 4.2.9. The protein concentration was determined using the Bradford method as described in 4.2.10. Buffers preparations are described in Appendix 4.

4.2.8 Crude enzyme production

The fermentation broth was centrifuged at 8000 rpm for 10 minutes to remove the cells, and the crude β–glucosidase in the culture supernatant was precipitated by adding solid ammonium sulphate $(NH_4)_2SO_4$ at 60% saturation. The solution was kept overnight at 0 – 4^{o}C. The precipitated proteins were collected by centrifugation at 26000 rpm for 20 minutes. The resultant pellet was re-dissolved in approximately 20 ml of 20 mM phosphate buffer (pH 7.0) and dialyzed against phosphate buffer for 2 days with 3 changes of buffer. The insoluble proteins were removed by centrifugation at 8000 rpm for 10 minutes.

The dialysis tubing (Sigma-Aldrich D-9777) with average flat width of 25 mm was initially treated by washing in running water for 3 – 4 hours to remove glycerin. This was followed by treatment with a 0.3% sodium sulfide solution at 80 ^{o}C for 1 minute to remove sulfur compounds. The tube was then washed with hot water (60 ^{o}C) for 2 minutes followed by acidification with 0.2% sulfuric acid. Finally, the dialysis tubing was thoroughly washed with hot water to remove the acid following the manufacturer's instructions.

4.2.9 Enzyme assay

β-glucosidase catalyses the hydrolysis of p-nitrophenol-β-glucoside to p-nitrophenol and glucose. Under alkaline conditions, the p-nitrophenolate anion absorbs light at 400 - 450 nm. The amount of enzyme present is therefore determined by measuring the amount of p-nitrophenolate anion produced in the reaction. β-Glucosidase was assayed using p-nitrophenyl- β-Dglucopyranoside (pNPG) as substrate as specified in Ghose and Bisaria (1987). Appropriately diluted enzyme samples of 25 µl were incubated with 25 µl of 10 mM pNPG in citrate buffer (0.05 M, pH 4.8) and 50 µl of citrate buffer (0.05 M, pH 4.8) at 40^{o}C for 15 minutes. The reaction was terminated by adding 100 µl of 0.2 M Na_2CO_3 solution. Appropriate blanks devoid of enzyme or substrate were also run in parallel to the enzyme assay. The colour developed due to liberation of p-Nitrophenol (pNP) was read in a spectrophotometer (Biochrom spec Libra S4. Serial no. – 105773, Biochrom Ltd, Cambridge England) at 405 nm and the amount of pNP liberated was calculated by comparing the reading corrected for blanks against a standard curve generated using varying concentrations of pNP (Appendix 9).

$$\text{pNPG} \xrightarrow{\beta\text{-glucosidase}} \text{pNP + D-Glucose}$$

One unit of β–glucosidase activity was defined as the amount of enzyme needed to liberate 1µM of p-Nitrophenol (pNP) per minute under the standard assay conditions.

4.2.10 Protein estimation

The protein concentration was determined using the Bradford method (Bradford 1976). 5 ml of the dye reagent was added to 100 µl of appropriately dilluted enzyme sample and was incubated for 5 minutes after which the absorbance was measured at 595 nm in a spectrophotometer (Biochrom spec Libra S4. Serial no. – 105773, Biochrom Ltd, Cambridge England). The amount of protein was determined by extrapolating from a bovine serum albumin (BSA) standard curve, constructed by using a solution containing 1 mg/ml BSA (Appendix 8). Bradford protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomasie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to proteins occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

4.2.11 Enzyme purification

4.2.11.1 Anion-exchange chromatography

Five milliliter (5 ml) of the dialyzed sample from section 4.2.8 was loaded onto the DEAE-Sephadex A-50 column equilibrated with phosphate buffer (pH 7). The column was eluted with a linear gradient (0.05M – 0.75 M) NaCl in phosphate buffer at pH 7.0 during washing. The activity of β-glucosidase in each fraction was determined. The active fractions were pooled together, concentrated using polyethylene glycol 1500 (BDH) and dialyzed extensively against phosphate buffer. The homogeneity of the purified β-glucosidase was checked by SDS-PAGE.

4.2.11.2 Determination of purity and molecular mass of β-glucosidase

Purity and molecular mass of the partially purified β-glucosidase was determined by SDS-PAGE using an appropriate molecular mass marker. The gel was stained using Sterling rapid silver stain following the manufacturer's instructions. After staining, photos of the bands were digitally captured.

4.2.11.3 Effect of pH on β-glucosidase activity

The effect of pH on the enzyme activity was studied within the pH 3.0 – 10.0 ranges:10 mM citrate buffer, pH 3.0 – 6.0; 10 mM phosphate buffer, pH 7.0 – 8.0; 10 mM glycine/NaOH buffer, pH 9.0 – 10.0. β-glucosidase activity was then assayed at each pH level. A graph of the β-glucosidase activity versus pH was drawn to determine optimum pH.

4.2.11.4 Effect of temperature on β-glucosidase activity

The effect of temperature on β-glucosidase activity was followed between 20 and 90^{o}C using the optimum pH. A graph of β-glucosidase activity versus temperature was plotted to determine optimum temperature.

4.2.11.5 Kinetic Constants (K_m and V_{max})

The substrate concentration was varied over the range of 0.01 – 0.14 g/ml of pNPG. The β-glucosidase activity was carried out as described in section 4.2.10. The kinetic constants, K_m and V_{max} for the enzyme were determined from a Lineweaver-Burk plot (double – reciprocal plot).

4.2.11.6 Thermostability studies of β-glucosidase

The thermostability of β-glucosidase was tested by incubating equal volumes (25 µl) of β-glucosidase enzyme at intervals of 10ºC for 30 min at a temperature range of 20 – 90ºC as described by Stauffer (1969). The various tubes were cooled to room temperature and treated as previously described in section 4.2.9. Plots of logarithm of β-glucosidase enzyme activity (LogV) versus reciprocal of temperature (K^{-1}) were carried out to determine Arrhenius plot of energy of activation of the purified enzyme. The residual activity of the enzyme was also determined.

4.2.11.7 Effect of Cations on β-glucosidase Activity

The effect of cations on the enzyme activity was determined. Exactly 25 µl of the enzyme and 5 µl of 0.02 M of the cations ($MgCl_2$, $CoCl_2$, $FeCl_3$, $CaCl_2$, $FeCl_2$ and $HgCl_2$) was incubated with 25 µl of pNPG and the enzyme activity was then assayed spectrophotometrically as described in section 4.2.10.

4.3 Results

4.3.1 Effects of pH on fungal and bacterial cellulase activity

Twenty one (21) fungal strains and eight (8) bacterial strains were screened to investigate the effects of pH on cellulase activity. All the fungal isolates used in this study had potential to degrade CMC (Appendix 31). The halo produced by hydrolysis of CMC is directly related to the region of action of cellulase activity, since the Congo dye only remain attached to regions where there are β-1,4-D-glucose bonds (Florencio *et al*., 2012). Example of pH on some of the fungal cellulase activities is shown in Figure 4.4. The results showed that the enzyme had a good activity within pH 4 – 6, with poorer activity towards alkaline pH when screened by Congo red method. Even though *A. niger* F321 showed maximum enzyme activity at pH 5.0, it could produce visible cellulase activity in the pH range of 4 .0 – 7.0 (Figure 4.4). Similarly, *D. arenaria* F200 also produced cellulase activity well in the pH range 4.0 – 5.0 with a decline within the alkaline pH (Appendix 31).

All the bacterial strains used in these studies had poor cellulase activity between pH 4 – 6 with the exception of *B. subtilis* 20 which had activity in both acidic and alkaline pH values (Appendix-32).

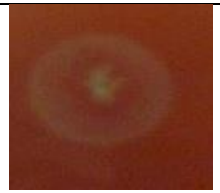
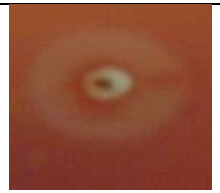
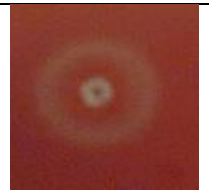
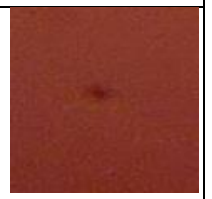
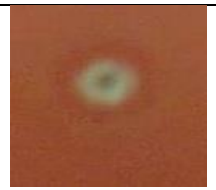
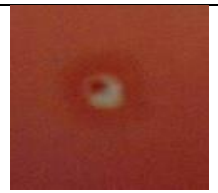
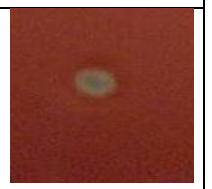
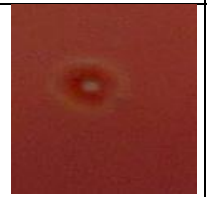
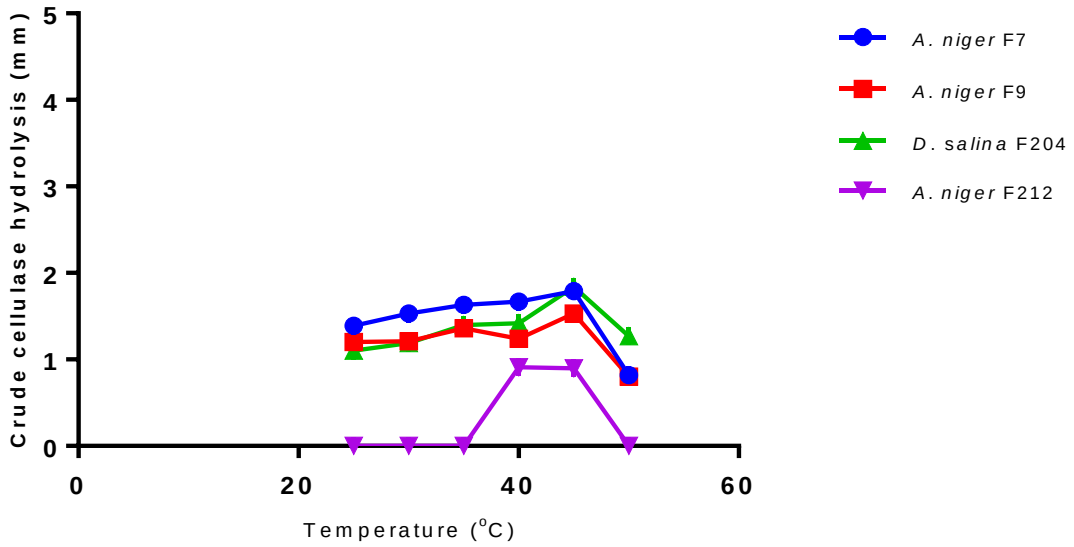
Strain/pH	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
A. niger F212						
A. niger F287						
A. niger F320						
A. niger F321						

Figure 4.4: Effect of pH on some fungal cellulase activity. Pictures were taken after 24 hours of incubation. See Appendix 31 for more details.

4.3.2 Effect of temperature on fungal cellulase activity

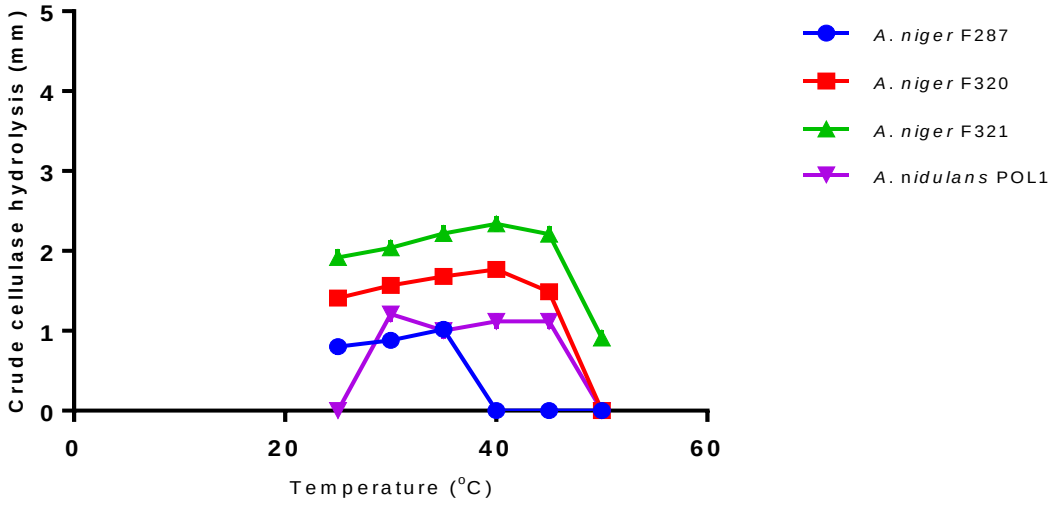
The 21 fungal strains used in this study were initially screened based on their ability to grow on CMC (Appendix 23). Efficient cellulase producing isolates were identified on the basis of their ability to hydrolyze CMC. The optimum temperature for cellulase hydrolysis was also determined. Although initial growth screening showed almost 100% of the fungal isolates had a good growth on CMC supplemented agar plates (Appendix 23), only 38.1% of the isolates had hydrolytic activity on CMC supplemented agar plates (Figure 4.5A and 4.5B; Appendix 30). The diameter of the halo showed the presence or absence of the cellulase production. Among the isolates that had hydrolytic activities were *A. niger* F7, *A. niger* F9, *A. niger* F212, *A. niger* F287, *A. niger* F320, *A. niger* F321, *A. nidulans* POL1 and *D. salina* F204.



Error bars represent the mean ± SEM (n = 3)

Figure 4.5A: Effect of temperature on cellulase production (zone of hydrolysis in mm) by some selected fungi after 48 hours of incubation on minimal medium. Majority of error mean were less than ± 0.09 and therefore bars were not place on points.

Extracellular cellulase activity of *Aspergillus niger* F321 had the highest activity on CMC with 2.22 ± 0.04 mm zone of hydrolysis with an optimum temperature of 45 ºC (Figure 4.5B).

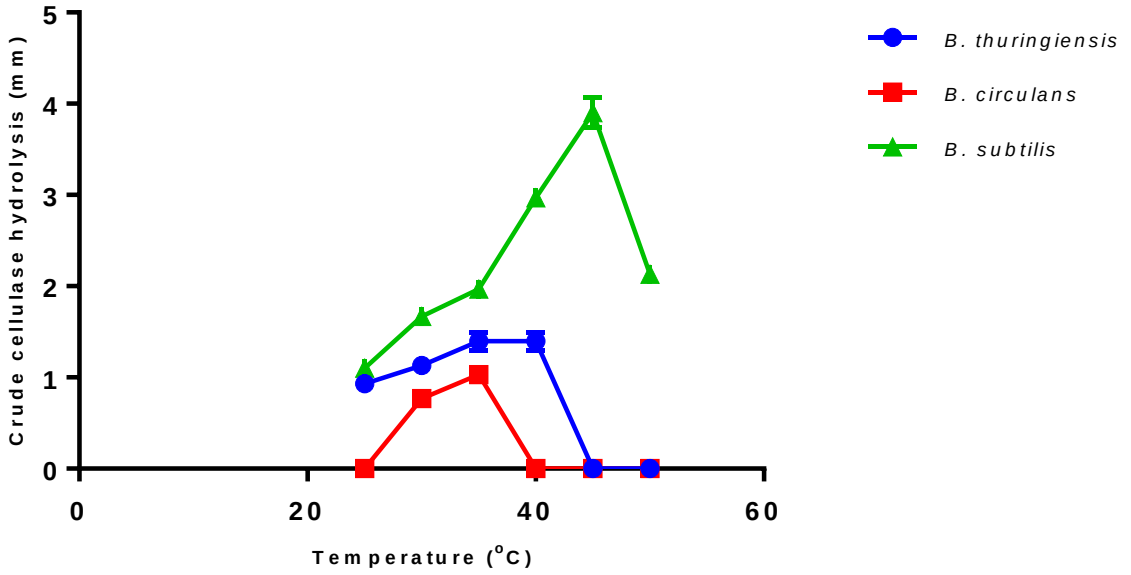


Error bars represent the mean ± SEM (n = 3)

Figure 4.5B: Effect of temperature on cellulase production (zone of hydrolysis in mm) by some selected fungi after 48 hours of incubation on minimal medium. Majority of error mean were less than ± 0.09 and therefore bars were not place on points.

4.3.3 Effect of temperature on bacterial cellulase production

Screening of bacteria for their cellulase activity was carried out at 25, 30, 35, 40, 45 and 50ºC. It is interesting to note that *B. subtilis* produced cellulases that had the highest zone of hydrolysis of 3.90 ± 0.17 mm with an optimum temperature of 45ºC (Figure 4.6, Appendix 34). Fifty seven percent (57%) of the bacterial strains screened did not show hydrolysis on CMC plates.



Error bars represent the mean ± SEM (n = 3)

Figure 4.6: Effect of temperature on crude cellulase activity (zone of hydrolysis in mm) of some selected *Bacillus* strains after 72 hours of incubation on minimal medium. Majority of error mean were less than ± 0.09 and therefore bars were not place on points.

4.3.4 Effect of carbon sources on fungal growth

The effects of carbon sources on fungal growth were investigated in these studies. Various carbon sources such as glucose, cellobiose, CMC and cellulose were used in the study. From the results, almost all the strains had a luxuriant growth on glucose supplemented agar plates (Appendix 33). The control plates (without carbon source) showed the absence of growth for all the isolates. Among the different carbon sources used, cellobiose was the second best after glucose with *A. nidulans* POL1 and *A. nidulans* G0281 having good growth on cellobiose supplemented agar plates (Appendix 33). The inoculation of *A. niger* 320 and *A. niger* 321 on cellobiose and CMC supplemented plates resulted in little growth on cellobiose supplemented plates and no growth on CMC supplemented plates, even

though they had a very good growth from previous studies on CMC agar plates (Appendix 23), to which a small concentration of glucose was added in the media.

4.3.5 Small Scale Shake Flask Fermentation of *P. pastoris* clones AN2612.2, AN0712.2, AN1551.2, AN2227.2 and AN1804.2

The study in this section focuses on the use of a simple and inexpensive shake flask fermentation process to determine the optimum duration for β-glucosidase enzyme production from recombinant *P. pastoris* clones AN2612.2, AN0712.2, AN1551.2, AN2227.2 and AN1804.2. Shake flask fermentation studies for the β-glucosidase enzyme production were carried out using 250 ml Erlenmeyer culture flask as described in section 2.4. Three shake flasks fermentations were operated in parallel for each experiment each containing 100 ml of medium. The *P. pastoris* 323 strain (without the β-glucosidase gene) was used as a control.

The effect of incubation time on β-glucosidase enzyme production was studied from 0 - 96 hours (Figure 4.8a-h Appendix 26 & 27) fermentation on Buffered Methanol-complex Medium (BMMY) using CMC as a substrate for enzyme hydrolysis.

The experiments in this section were carried out to monitor the growth of the engineered FGSC strains in BMMY medium and to determine the β-glucosidase enzyme production yield over a period of time. The recombinant *P. pastoris* clones AN2612.2, AN0712.2, AN1551.2, AN2227.2 and AN1804.2 clones respectively were inoculated into 25 ml of Buffered Minimal Glycerol (BMGY) medium in a 100 ml conical flask. Cell cultures were incubated on a shaker at 30 ^{o}C and 150 rpm for 18 hours to grow in the BMGY medium before induction. When the culture concentration reached an OD_{600} = 2 the cells were harvested by centrifuging at 24 ^{o}C and 5000 rpm for 5 minutes. The cell pellets were then suspended in 90 ml of BMMY medium until OD_{600} = 1 to induce expression. The culture was incubated on a shaker at 150 rpm and 30 ^{o}C for 96 hours (4 days). The cell culture was

induced every 24 hours after the start of cultivation with methanol to 0.5% of the culture to compensate for methanol loss caused by evaporation and uptake by cells. Samples were taken after 0, 3, 6, 9, 12, 15, 18, 24, 48, 72 and 96 hours' time points for analysis as described in section 4.2.7.

Figure 4.7a (i) and Figure 4.7a (ii) show the growth curve and the β-glucosidase enzyme activity respectively of *P. pastoris* with accession AN0712.2. The maximum activity of β-glucosidase hydrolysis on CMC supplemented agar plates was observed at 48 and 72 hours and it was found to be 11.11 ± 1.02 mm and 11.11 ± 1.39 mm respectively. The maximum cell growth was observed at 96 hours and it was found to be 8.59 ± 0.06 Log10 CFU/ml.

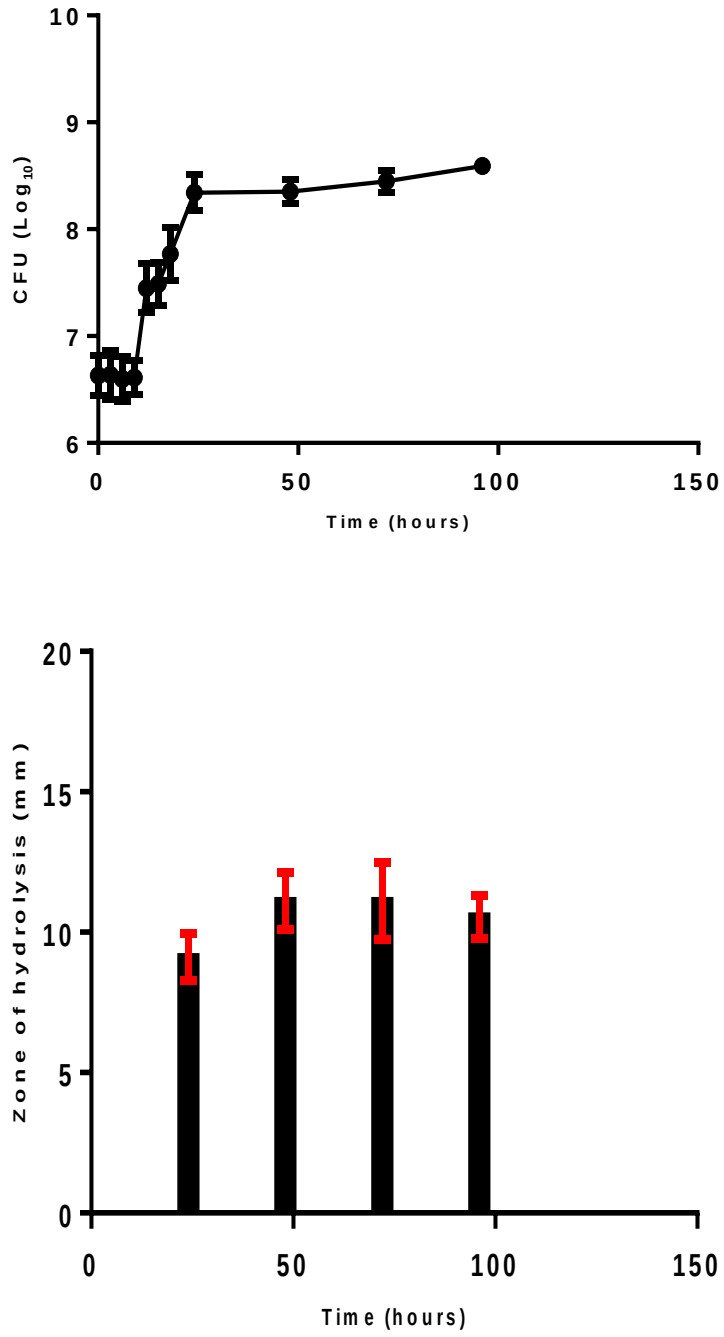


Figure 4.7a: Colony forming unit (i) and zone of hydrolysis (ii) chart of *P. pastoris* with accession AN0712.2 cultivated on BMMY. Results are the average of three replicates and bars indicate standard deviation of three replicates.

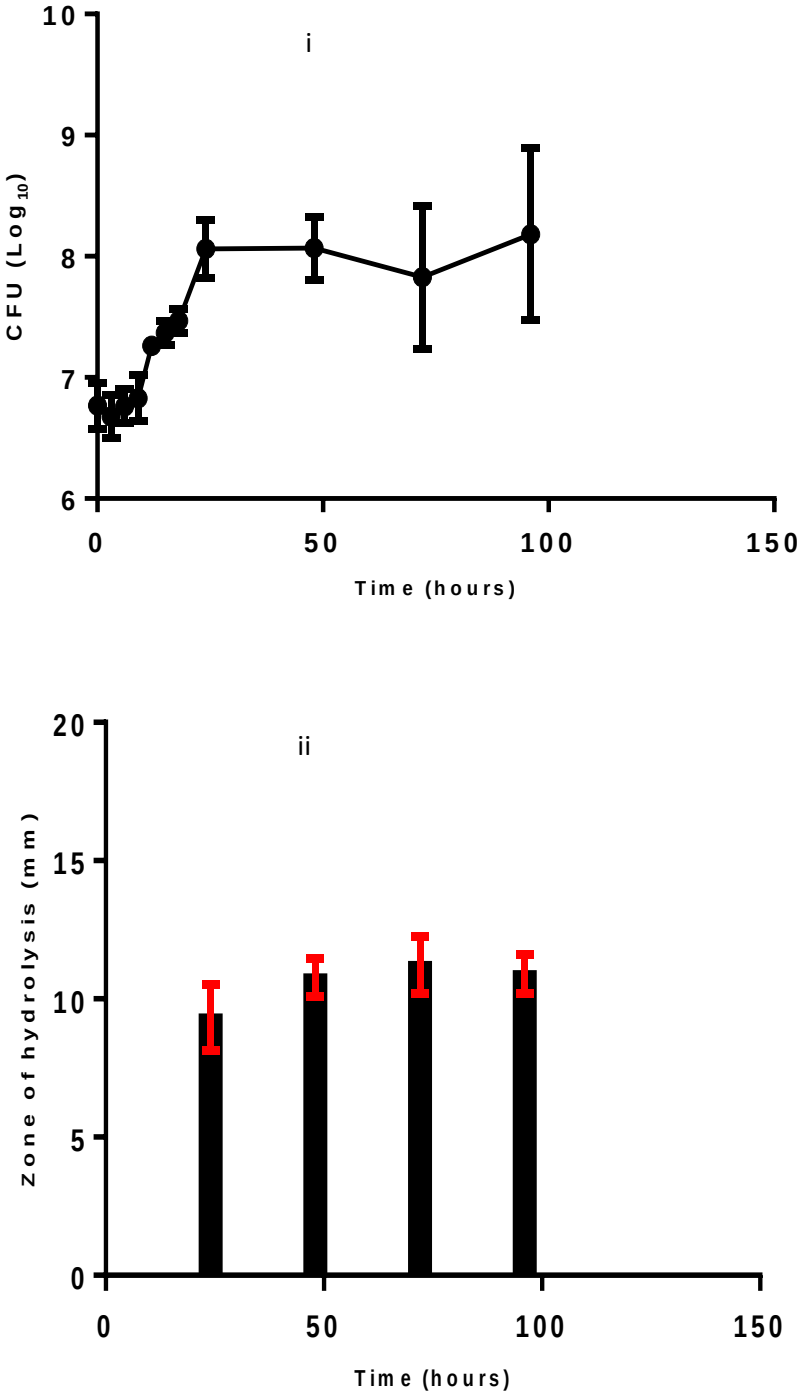


Figure 4.7b: Colony forming unit (i) and zone of hydrolysis (ii) chart of *P. pastoris* with accession number AN1551.2 cultivated on BMMY. Results are the average of three replicates and bars indicate standard deviation of three replicates.

Figure 4.7b (i) and Figure 4.7b (ii) show the growth curve and the β-glucosidase enzyme activity respectively of *P. pastoris* with accession AN1551.2. The maximum activity of β-glucosidase hydrolysis on CMC supplemented agar plates was observed at 72 hours and it was found to be 11.22 ± 1.02 mm. The cell growth curve shows a decrease in growth at 72 hours with an increase at 96 hours. The maximum cell growth was observed at 96 hours and it was found to be 8.18 ± 0.71 Log10 CFU/ml.

Figure 4.7c (i) and Figure 4.7c (ii) show the growth curve and the β-glucosidase enzyme activity respectively of *P. pastoris* with accession AN1804.2. The maximum activity of β-glucosidase hydrolysis on CMC supplemented agar plates was also observed at 72 hours and it was found to be 11.56 ± 1.71 mm. The maximum cell growth was observed at 96 hours and it was found to be 8.60 ± 0.29 Log10 CFU/ml.

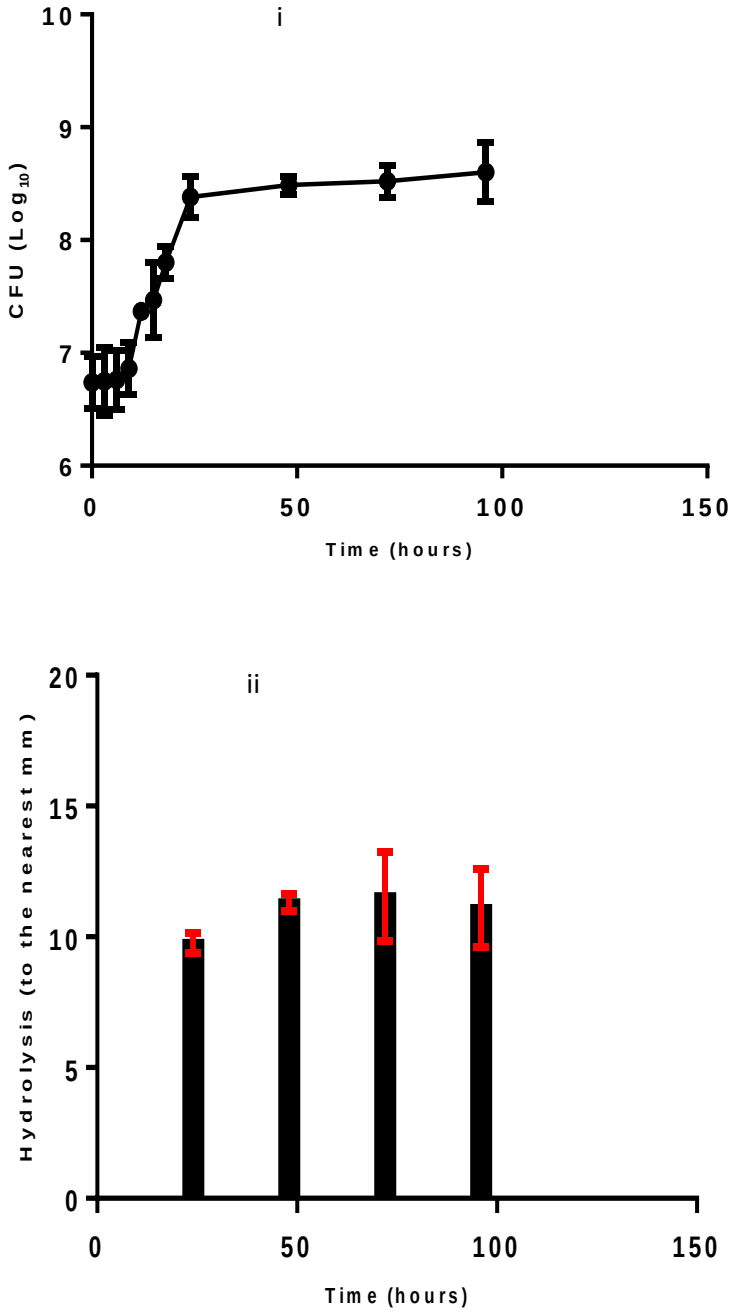


Figure 4.7c: Colony forming unit (i) and zone of hydrolysis (ii) chart of *P. pastoris* clone with accession number AN1804.2 cultivated on BMMY. Results are the average of three replicates and bars indicate standard deviation of three replicates.

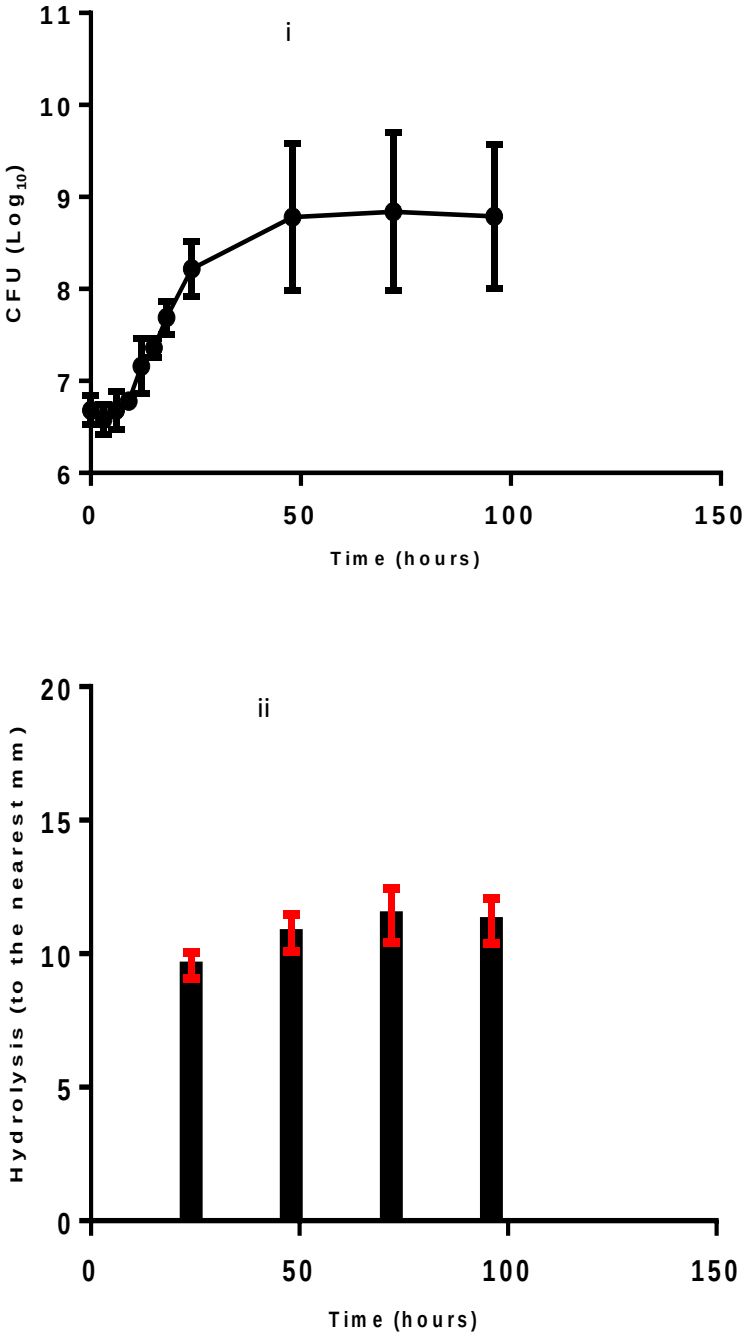


Figure 4.7d: Colony forming unit (i) and zone of hydrolysis (ii) chart of *P. pastoris* clone with accession number AN2227.2 cultivated on BMMY. Results are the average of three replicates and bars indicate standard deviation of three replicates.

Figure 4.7d (i) and Figure 4.7d (ii) show the growth curve and the β-glucosidase enzyme activity respectively of *P. pastoris* with accession AN2227.2. The maximum activity of β-glucosidase hydrolysis on CMC supplemented agar plates was also observed at 72 hours and it was found to be 11.44 ± 1.02 mm. The maximum cell growth was observed at 72 hours and it was found to be 8.84 ± 0.86 Log10 CFU/ml. This is the only strain that had its maximum enzyme activity is corresponding with maximum cell growth at the same time.

Figure 4.7e (i) and Figure 4.7e (ii) show the growth curve and the β-glucosidase enzyme activity respectively of *P. pastoris* with accession AN2612.2. The maximum cell growth was observed at 96 hours and it was found to be 8.74 ± 0.07 Log10 CFU/ml while the maximum activity of β-glucosidase hydrolysis on CMC supplemented agar plates was observed at 72 hours and it was found to be 11.22 ± 1.35 mm.

Finally, Figure 4.7f (i) and Figure 4.7f (ii) show the growth curve and the β-glucosidase enzyme activity respectively of the wild type *P. pastoris* 323 which serves as a negative control. The maximum cell growth was also observed at 96 hours and it was found to be 8.70 ± 0.06 Log10 CFU/ml. There was no activity recorded for the wild-type *P. pastoris.*

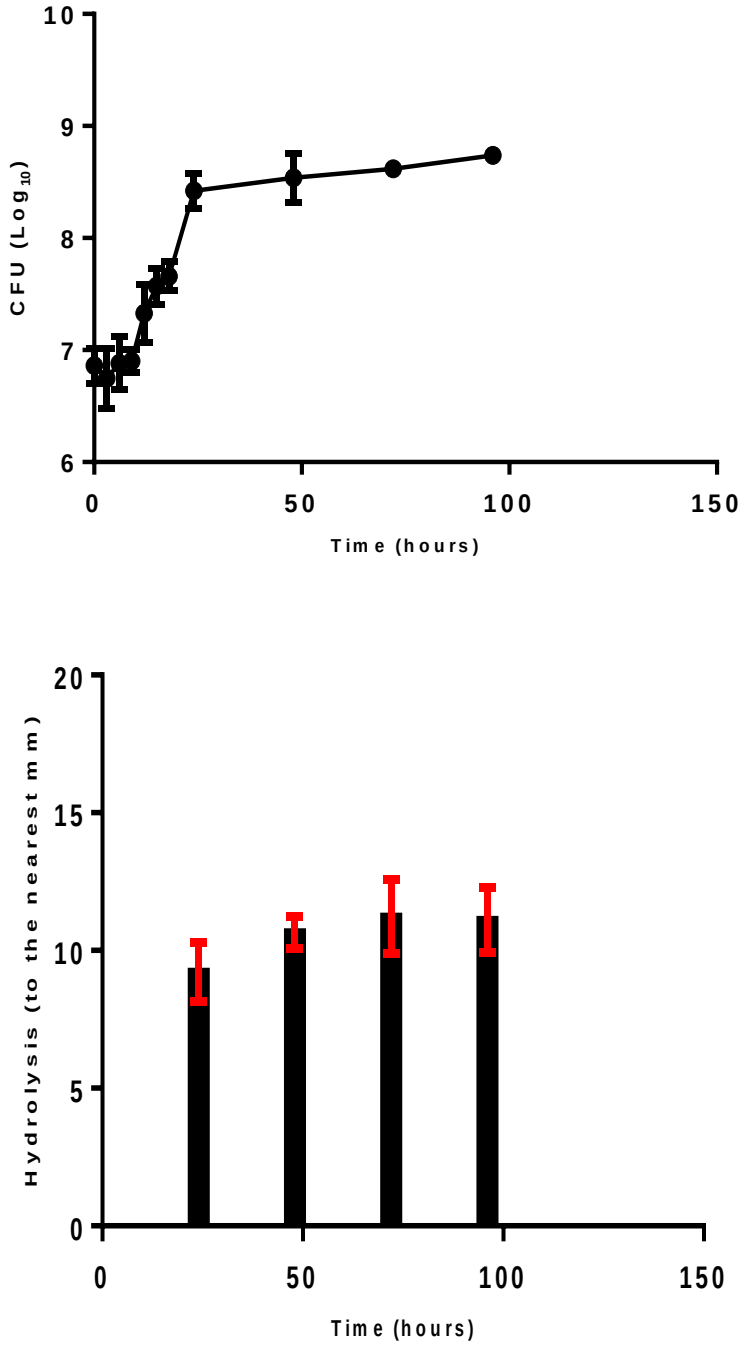


Figure 4.7e: Colony forming unit (i) and zone of hydrolysis (ii) chart of *P. pastoris* clone with accession number AN2612.2 cultivated on BMMY. Results are the average of three replicates and bars indicate standard deviation of three replicates.

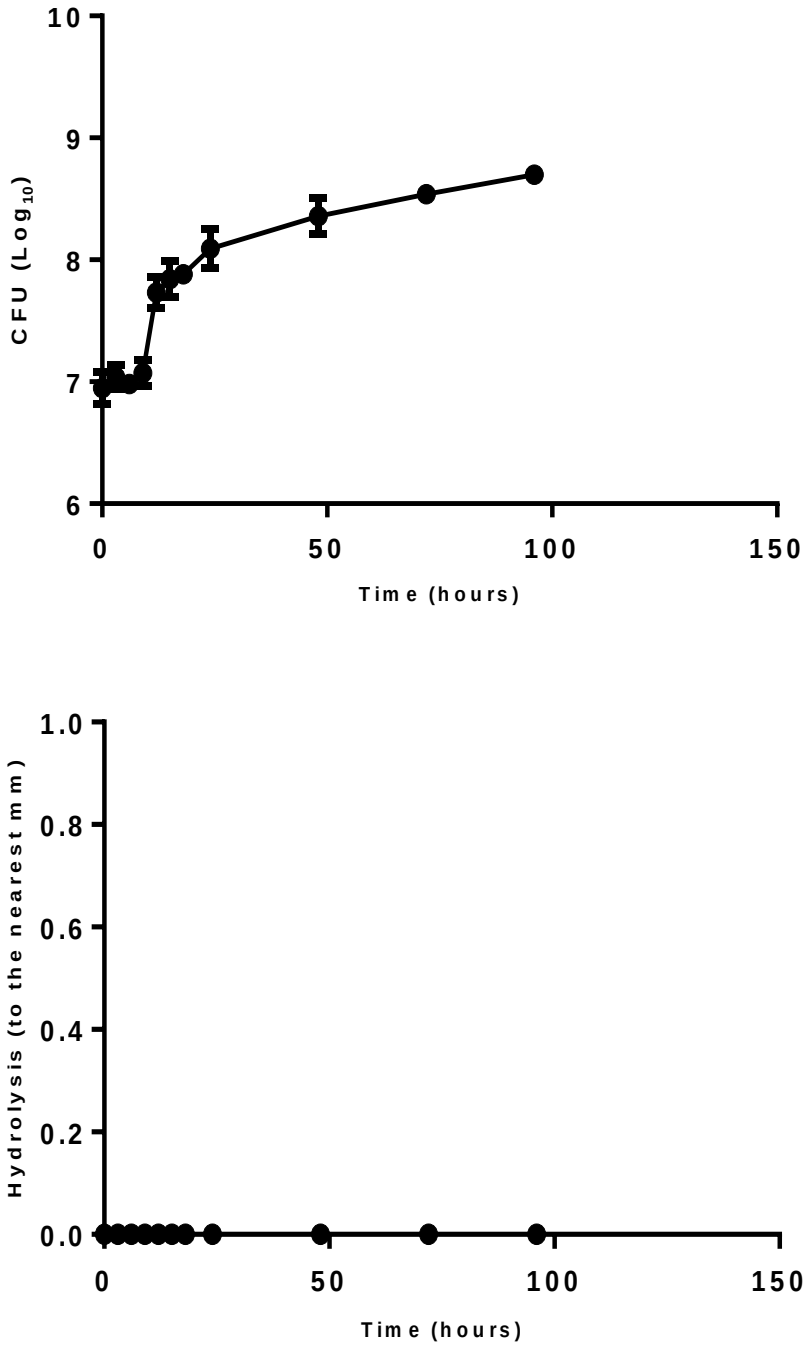


Figure 4.7f: Colony forming unit (i) and zone of hydrolysis (ii) chart of the wild type control *P. pastoris* 323 strains cultivated on BMMY. Results are the average of three replicates and bars indicate standard deviation of three replicates.

4.3.6 Small scale expression of the AN2227.2 and AN1804.2 recombinant β-glucosidase

Chapter 4 (Section 4.3.5) describes the optimization of expression of five *A. nidulans* clones in *P. pastoris*. A characterization of two *A. nidulans* β-glucosidase clones already sub cloned into pPICZ was then attempted. Figure 4.8 (Appendix 10) shows the changes in crude protein concentration of *A. nidulans* AN2227.2 and AN1804.2 β-glucosidase in Buffered Methanol Complex Medium (BMMY). In both *A. nidulans* AN2227.2 and AN1804.2 inoculated media, β-glucosidase protein concentration increased until it reached optima of 0.56 mg/ml for AN2227.2 and 0.67 mg/ml for AN1804.2 after 48 hours and the concentration subsequently declined.

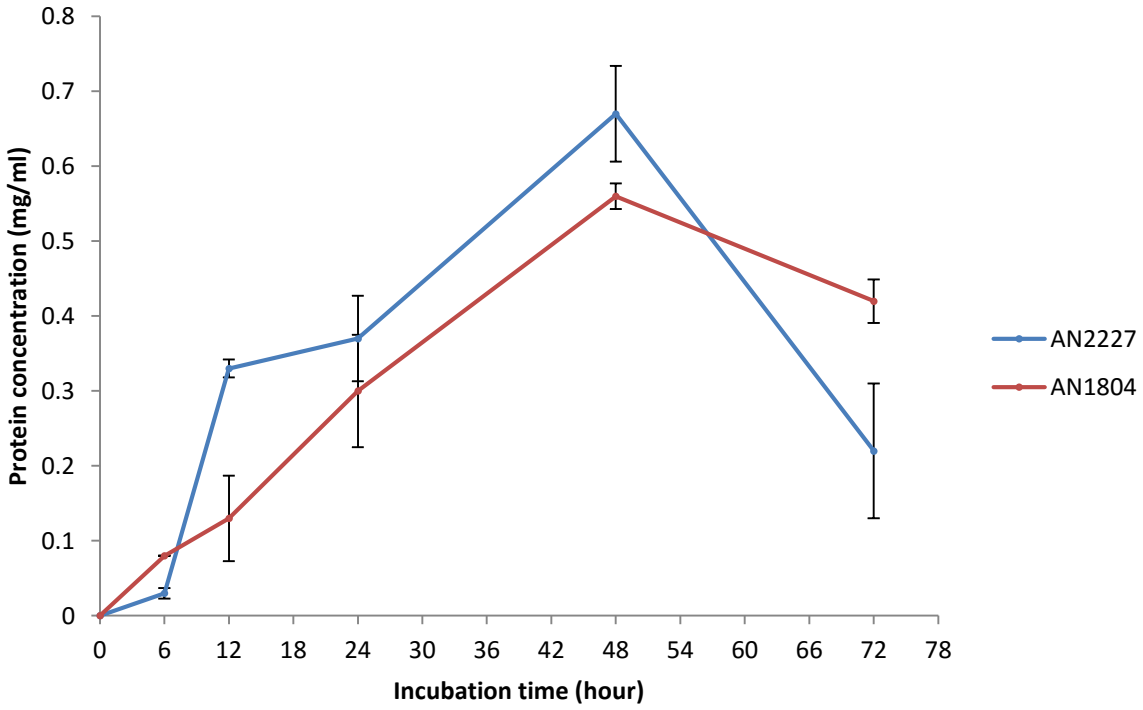


Figure 4.8: Time course for crude protein indicative of potential β-glucosidase production by *A. nidulans* AN2227.2 and AN1804.2

Error bars represent the mean ± SEM (n = 3)

Figure 4.9 (Appendix 11) shows the time course of *A. nidulans* AN2227.2 and AN1804.2 β-glucosidase activity on p-nitrophenyl-β-Dglucopyranoside (pNPG) as substrate. *A.*

nidulans AN2227.2 β-glucosidase had a very low enzyme activity on pNPG with an optimum activity of 0.54 µmole/ml/min on the second day (48th hour). *A. nidulans* AN1804.2 β-glucosidase was very active on pNPG with an increase in activity until it reached an optimum of 1.78 µmole/ml/min on the second day (48th hour) and subsequently declined.

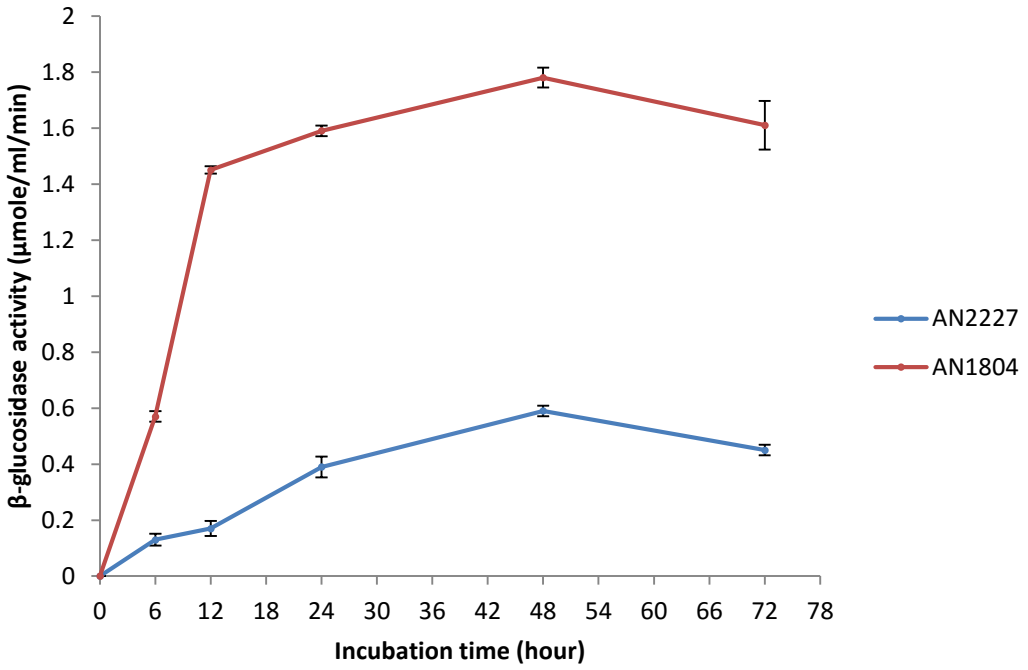


Figure 4.9: Time course for β-glucosidase activity by *A. nidulans* AN2227.2 and AN1804.2

Error bars represent the mean ± SEM (n = 3)

A pilot run conducted to establish the appropriate percentage of ammonium sulphate saturation to precipitate β-glucosidase enzyme from both *A. nidulans* AN2227.2 and AN1804.2 showed that the enzyme suspension was precipitated between 40 – 80%. At this stage of β-glucosidase enzyme purification, a 1.02 fold purification was achieved for *A. nidulans* AN2227.2 β-glucosidase over the crude extract (Table 4.1). Also, a 1.17 fold

purification was achieved for *A. nidulans* AN1804.2 β-glucosidase over the crude extract with $(NH_4)_2SO_4$ (Table 4.2).

Table 4.1: Purification profile of β-glucosidase from *A. nidulans* AN2227.2

	Total protein (mg)	**Total activity (µmole/ml/min)**	**Specific activity (µmole/ml/min/ mg protein)**	**Purification (fold)**	**Yield (%)**
Culture filtrate (crude)	0.31	0.66	2.13	1	100
$(NH_4)_2SO_4$ precipitation	0.17	0.37	2.17	1.02	56.06
Dialysis	0.03	0.12	4.00	1.88	18.18
DEAE-Sephadex A-50	0.02	0.11	5.50	2.58	16.67

Table 4.2: Purification profile of β-glucosidase from *A. nidulans* AN1804.2

	Total protein (mg)	**Total activity (µmole/ml/min)**	**Specific activity (µmole/ml/min/ mg protein)**	**Purification (fold)**	**Yield (%)**
Culture filtrate (crude)	0.88	1.59	1.81	1	100
$(NH_4)_2SO_4$ precipitation	0.51	1.08	2.12	1.17	67.92
Dialysis	0.26	0.81	3.12	1.72	50.94
DEAE-Sephadex A-50	0.13	0.53	4.08	2.25	33.33

After ammonium sulphate precipitation, the β-glucosidases were dialyzed against 20 mM phosphate buffer (pH 7.0) with a 1.88 fold purification over the crude extract for *A. nidulans* AN2227.2 β-glucosidase (Table 4.1) and 1.72 fold purification over crude extract for *A. nidulans* AN1804.2 β-glucosidase (Table 4.2).

4.3.6.1 Anion-exchange chromatography

Figure 4.10 (Appendix 12) shows the anion exchange chromatography elution profile of *A. nidulans* AN2227.2 β-glucosidase. The elution profile resulted in a single broad peak (fraction 9 – 19). The active fractions (9 – 15) were pulled together and dialysed against the phosphate buffer. The peak produced a final purification of 2.58 fold over the crude extract for *A. nidulans* AN2227.2 β-glucosidase (Table 4.1). The anion exchange chromatography elution profile of *A. nidulans* AN1804.2 β-glucosidase is shown in Figure 4.11 (Appendix 13). Similarly, the elution profile resulted in a single broad peak (fractions 10 – 16) which were pulled together and dialysed against the phosphate buffer. The collated fractions produced a final purification of 2.25 fold over the crude extract for *A. nidulans* AN1804.2 β-glucosidase (Table 4.2).

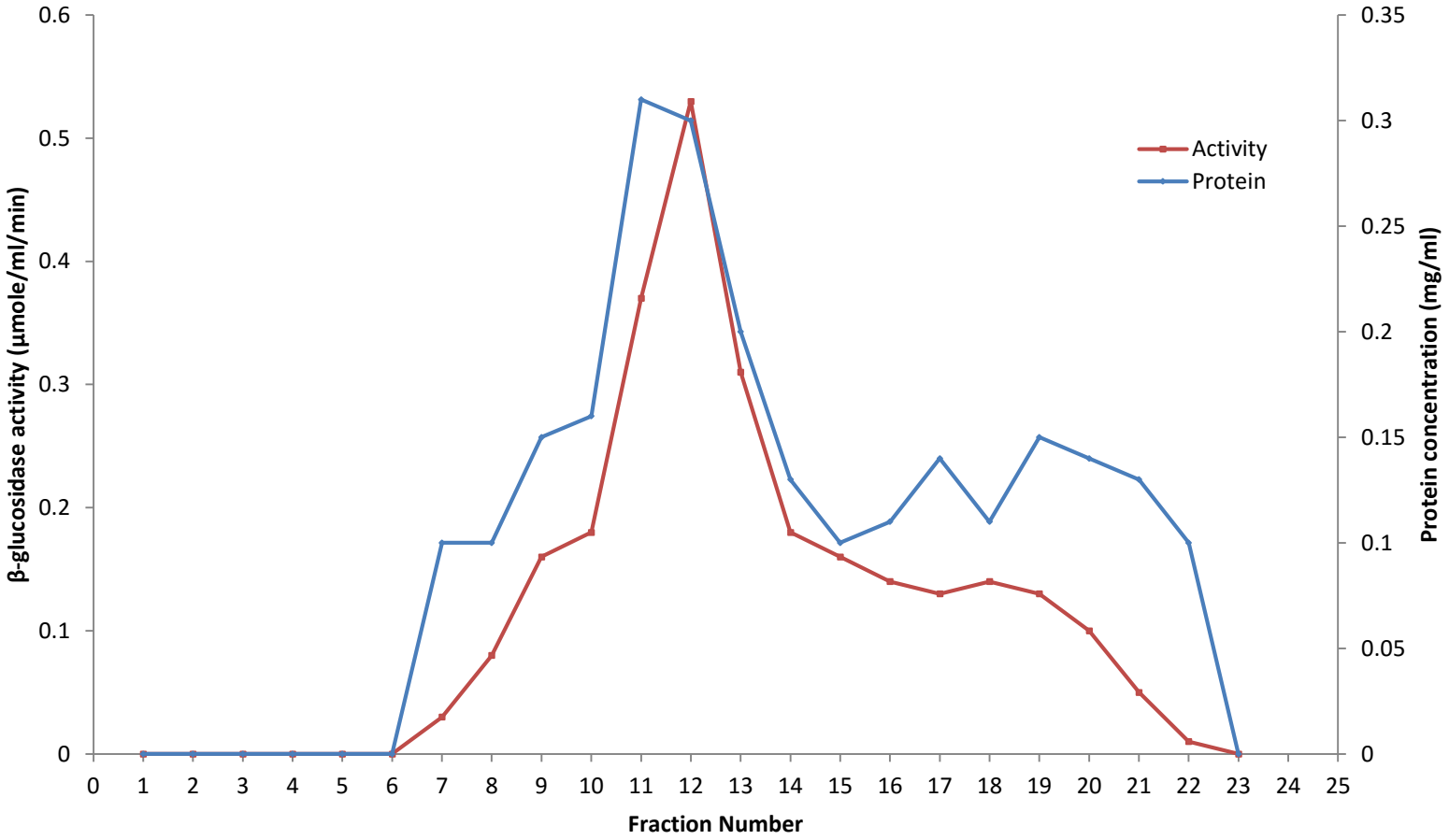


Figure 4.10: Elution profile of *A. nidulans* AN2227.2 β-glucosidase from DEAE-sephadex A-50 anion exchange column

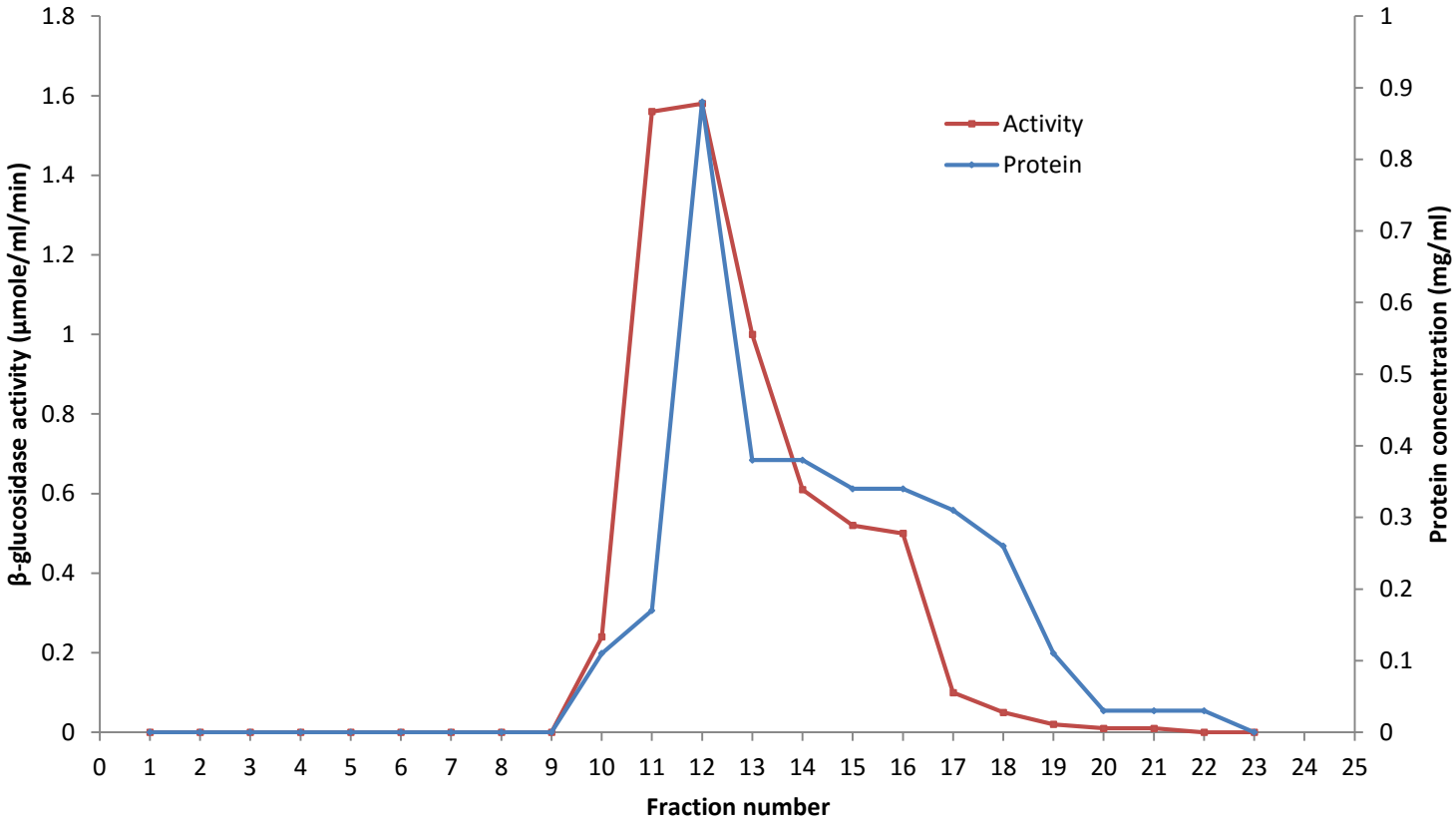


Figure 4.11: Elution profile of *A. nidulans* AN1804.2 β-glucosidase from DEAE-Sephadex A-50 anion exchange column

4.3.6.2 Determination of purity and molecular mass

The purity and molecular mass of the partially purified β-Glucosidases from *A. nidulans* AN2227.2 and AN1804.2 were determined by SDS-PAGE and stained using Sterling rapid silver stain. Figure 4.12 shows the purified *A. nidulans* AN2227.2 β-glucosidase enzyme moved homogenously as a single band on the polyacrylamide gel. The β-glucosidase enzyme moved at the same speed corresponding to an estimated molecular weight of 48 kDa as S6-0024 BLUeye prestained protein ladder (Tris-Glycine 4 – 20%) with an estimated protein level of 5 ng/band.

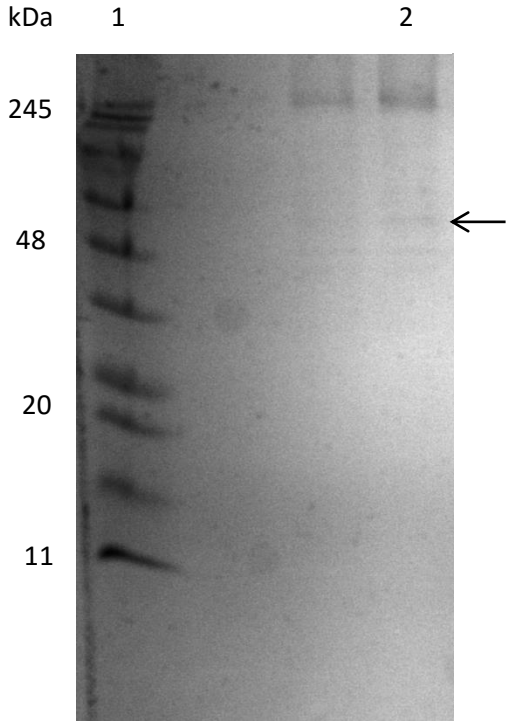


Figure 4.12: SDS-PAGE of *A. nidulans* AN2227.2 β-glucosidase. Lane 1: molecular weight standards. Lane 2: purified enzyme. The gel was stained with Sterling rapid silver stain

Figure 4.13 shows the purified *A. nidulans* AN1804.2 β-glucosidase enzyme also moved homogenously as a single band on the SDS polyacrylamide gel. The β-glucosidase enzyme moved at the same speed corresponding to an estimated molecular weight of 100 kDa as

S6-0024 BLUeye prestained protein ladder (Tris-Glycine 4 – 20%) with an estimated protein level of 300 ng/band.

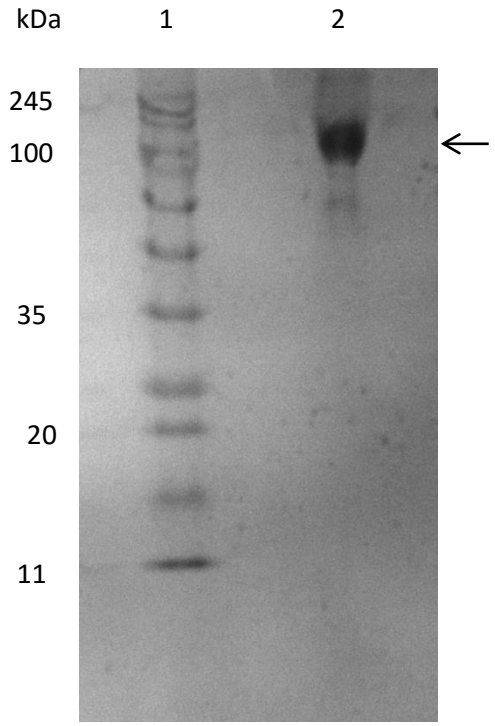


Figure 4.13: SDS-PAGE of *A. nidulans* AN1804.2 β-glucosidase. Lane 1: molecular weight standards. Lane 2: purified enzyme. The gel was stained with Sterling rapid silver stain

4.3.6.3 Effect of pH on β-glucosidase activity

The effect of pH on the β-glucosidase enzyme activity was studied within the pH 3.0 – 10.0 ranges. Figure 4.14 and 4.15 (Appendix 14) show the pH profile of *A. nidulans* AN2227.2 and AN1804.2 β-glucosidase respectively. The *A. nidulans* AN2227.2 β-glucosidase, but especially AN1804.2 had strikingly broad pH ranges and the enzymes were most active at a pH 6.0 (AN2227.2) and pH 5.5 (AN1804.2). The activity decreased markedly beyond pH 8.0 for AN2227.2.

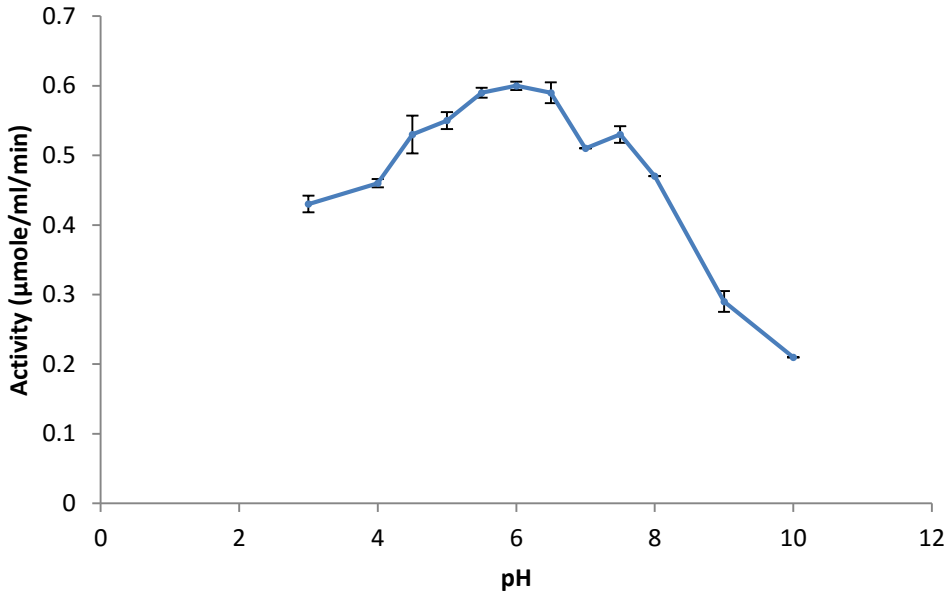


Figure 4.14: Effect of pH on AN2227.2 β-glucosidase activity

Error bars represent the mean ± SEM (n = 3)

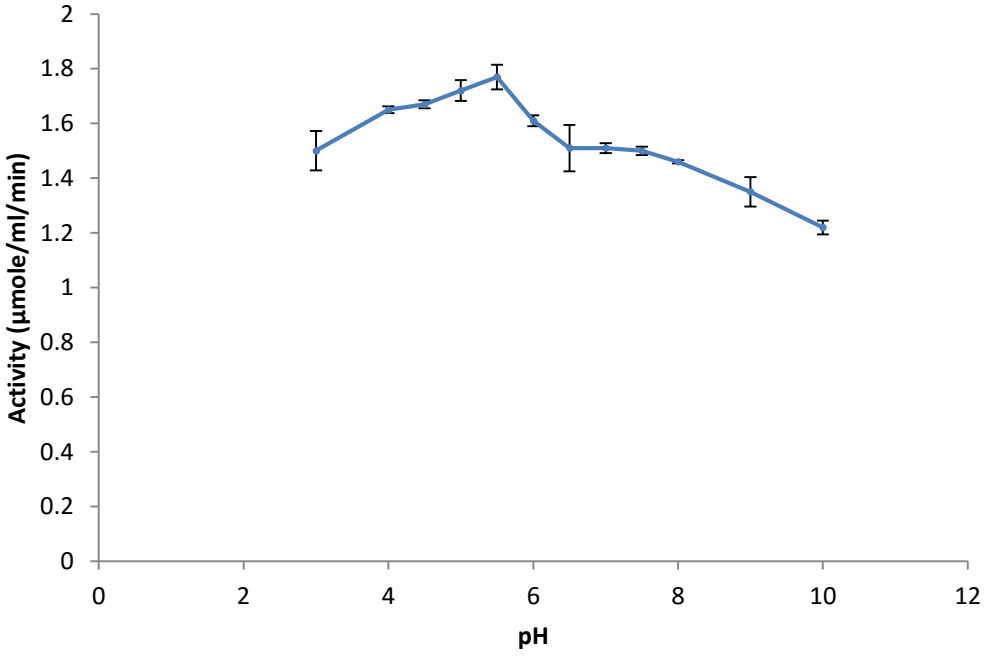


Figure 4.15: Effect of pH on AN1804.2 β-glucosidase activity

Error bars represent the mean ± SEM (n = 3)

4.3.6.4 Effect of temperature on β-glucosidase activity

Figure 4.16 and Figure 4.17 (Appendix 15) shows the temperature profile of *A. nidulans* AN2227.2 and AN1804.2 β-glucosidase respectively. β-glucosidase activity was followed between 20 and 90ºC using the optimum pH of 6.0 for AN2227.2 and pH 5.5 for AN1804.2. *A. nidulans* AN2227.2 β-glucosidase enzyme was optimally active at 40 ºC. The activity however dropped sharply above 50 ºC.

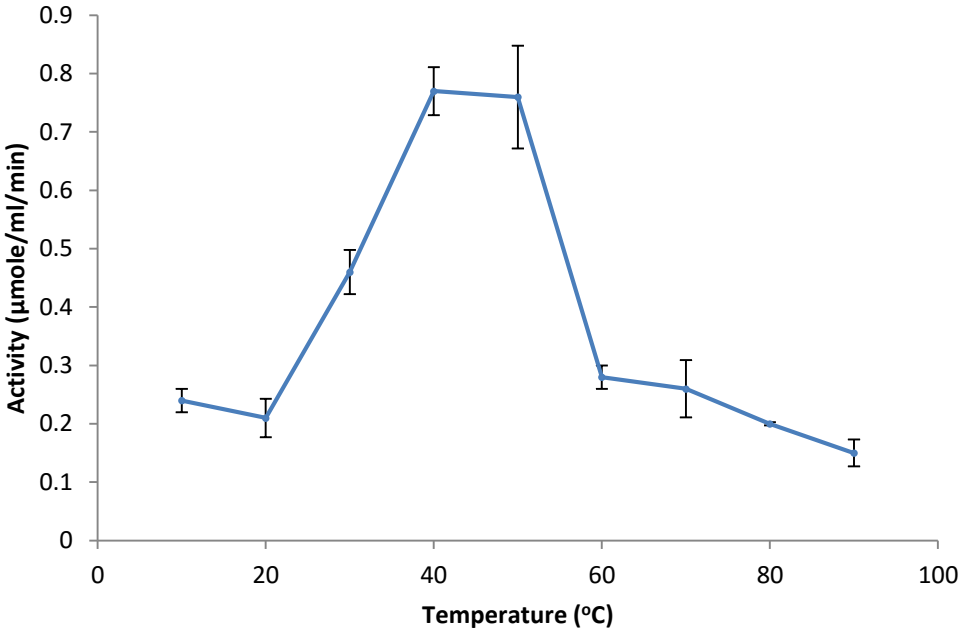


Figure 4.16: Effect of temperature on AN2227.2 β-glucosidase activity

Error bars represent the mean ± SEM (n = 3)

Using a pH of 5.5 as the optimum pH for *A. nidulans* AN1804.2 β-glucosidase, the enzyme was optimally active at 50 ºC (Figure 4.17). The purified enzyme produced a very broad temperature range of activity and good activity was even observed at 70 ºC. The activity however dropped sharply at temperatures above 70 ºC.

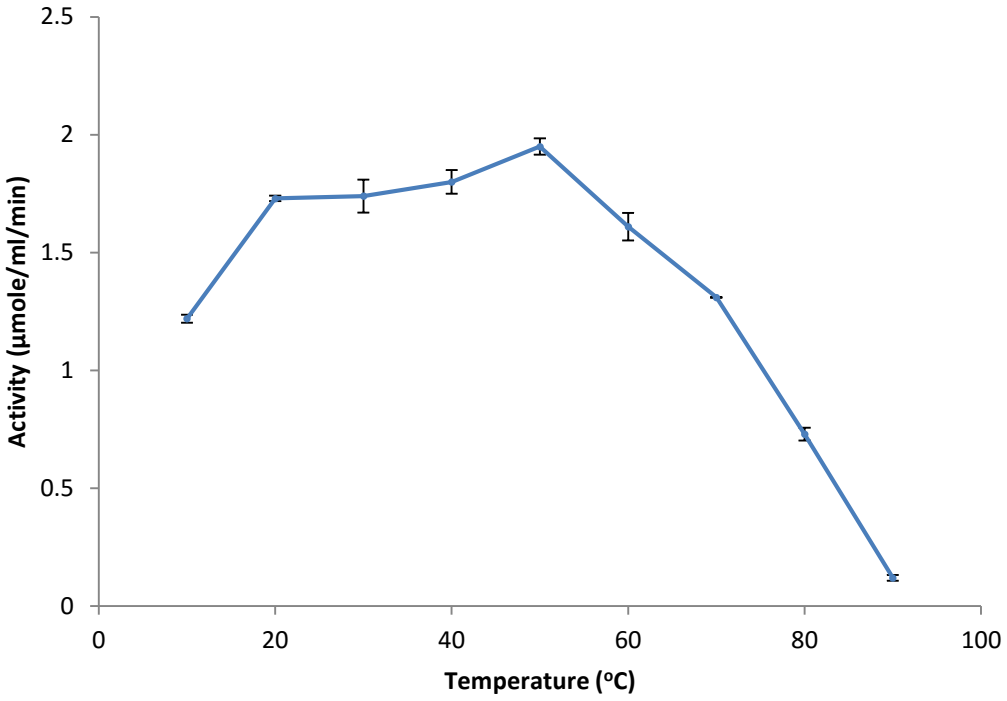


Figure 4.17: Effect of temperature on AN1804.2 β-glucosidase activity

Error bars represent the mean ± SEM (n = 3)

4.3.6.5 Thermostability studies of β-glucosidase

Thermostability studies were conducted using the optimum pH of 6.0 for *A. nidulans* AN2227.2 β-glucosidase and pH 5.5 for *A. nidulans* AN1804.2 β-glucosidase. Figure 4.18 (Appendix 16) shows the temperature stability profile of *A. nidulans* AN2227.2 β-glucosidase. The β-glucosidase stability was estimated up to 90 °C. *A. nidulans* AN2227.2 β-glucosidase was active at 30 °C and retained about 46.43% activity (Figure 4.19; Appendix 17).

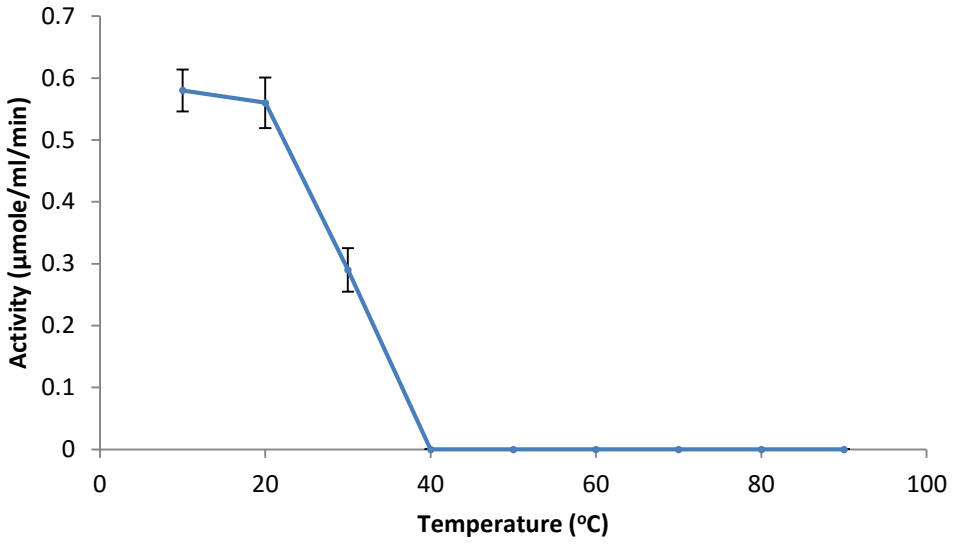


Figure 4.18: Thermostability of AN2227.2 β-glucosidase

Error bars represent the mean ± SEM (n = 3)

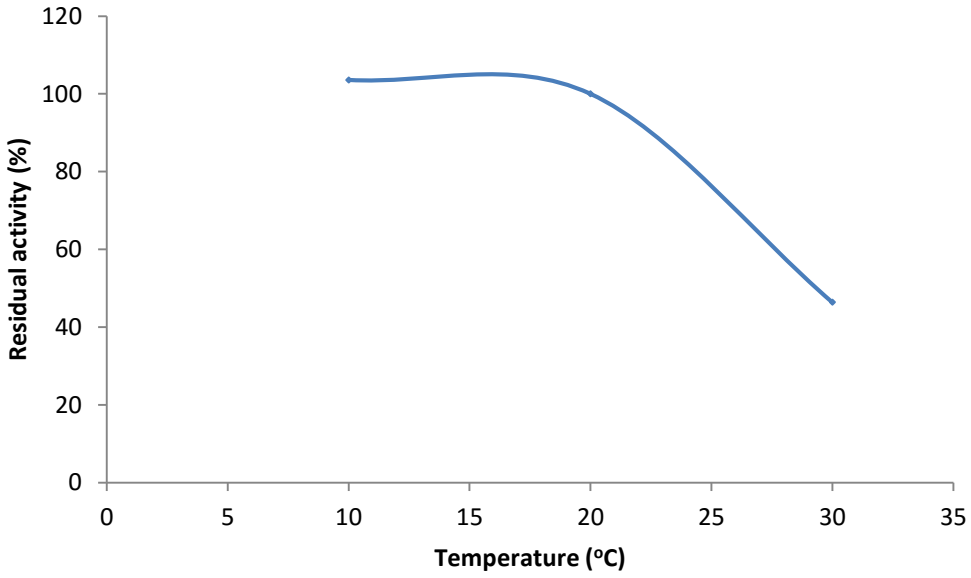


Figure 4.19: Residual activity of AN2227.2 β-glucosidase

Residual activity is an expression to indicate that there is loss in the original activity after the enzyme is subjected to different temperature pretreatment.

Figure 4.20 (Appendix 18) shows the temperature stability profile of *A. nidulans* AN1804.2 β-glucosidase. The β-glucosidase stability was also estimated up to 90 ^{o}C. The temperature dependent study revealed a remarkable broad activity in the range of 10 – 50 ^{o}C. However, the enzyme completely lost activity at temperatures above 60 ^{o}C. The enzyme was active at 50 ^{o}C and retained about 94.19% activity (Figure 4.21; Appendix 19).

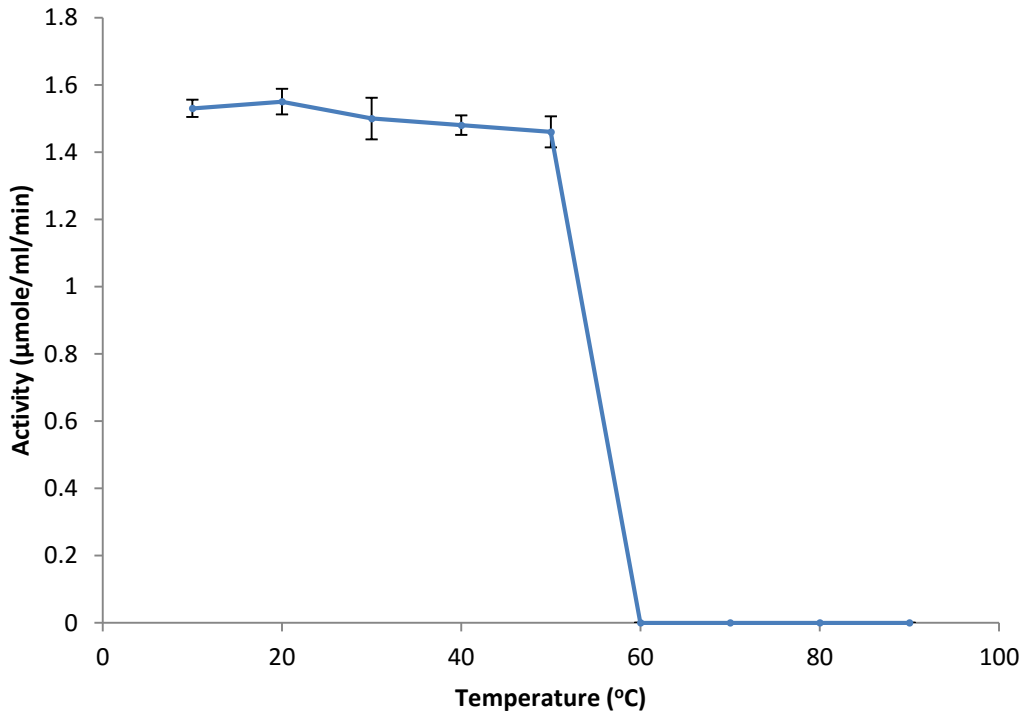


Figure 4.20: Thermostability of AN1804.2 β-glucosidase

Error bars represent the mean ± SEM (n = 3)

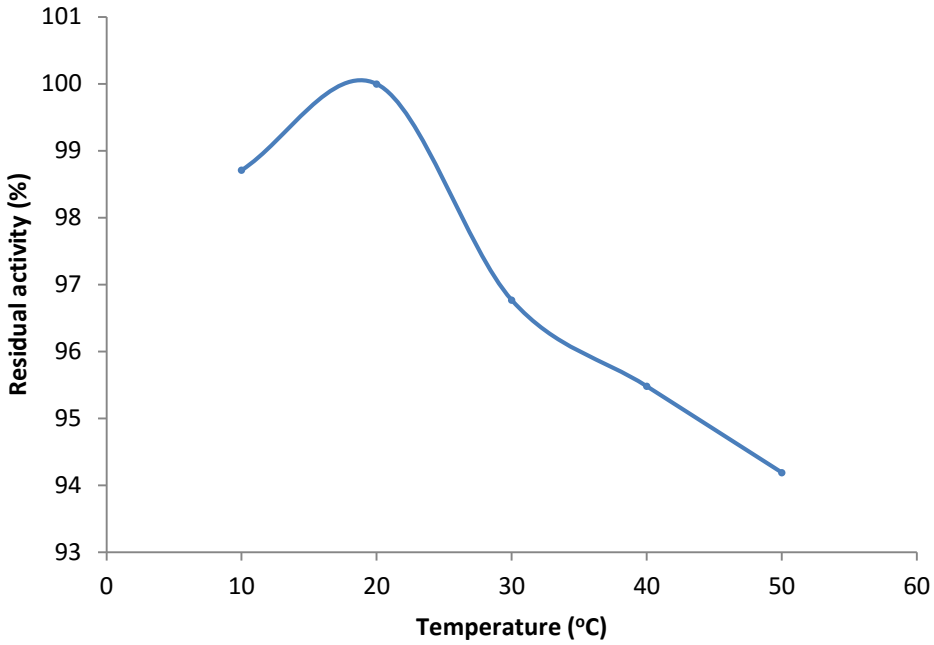


Figure 4.21: Residual activity of AN1804.2 β-glucosidase

4.3.6.6 Kinetic Constants (K_m and V_{max})

The effect of substrate concentration in this study was varied over the range of 0.01 – 0.14 g/ml of pNPG. Km and Vmax for the hydrolysis of pNPG by β-glucosidases was determined and data analyzed from Lineweaver-Burk plots. The optimum pH of 6.0 for *A. nidulans* AN2227.2 β-glucosidase and pH 5.5 for *A. nidulans* AN1804.2 β-glucosidase while the optimum temperature of 40 ^{o}C and 50 ^{o}C respectively was used for the enzymes. Figure 4.22 and 4.23 (Appendix 20) shows the effect of substrate concentration on reaction velocity at constant enzyme concentration and the double reciprocal plot of *A. nidulans* AN2227.2 β-glucosidase activity. Figure 4.22 shows that the enzyme activity increased with substrate concentration until all the enzyme was saturated at a substrate concentration of 100 mg/ml.

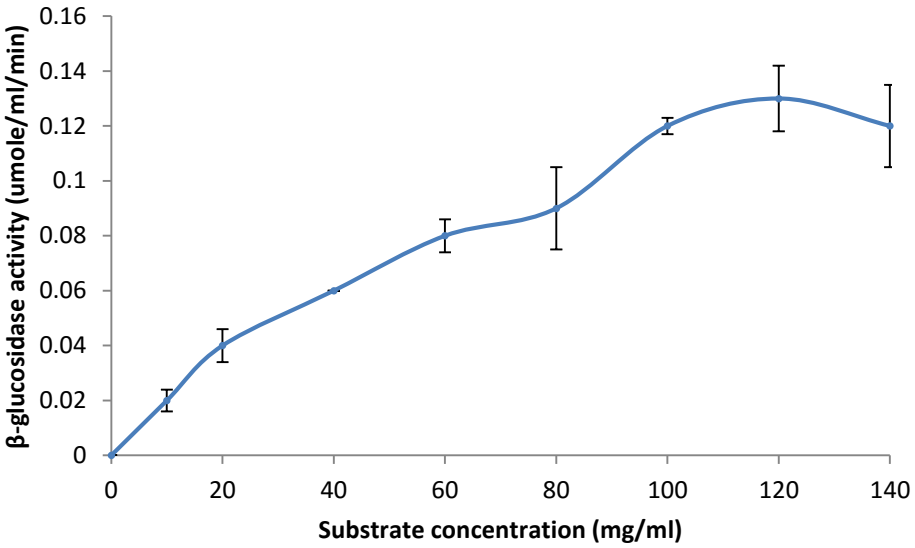


Figure 4.22: Effect of substrate concentration on the activity of *A. nidulans* AN2227.2 β-glucosidase

Error bars represent the mean ± SEM (n = 3)

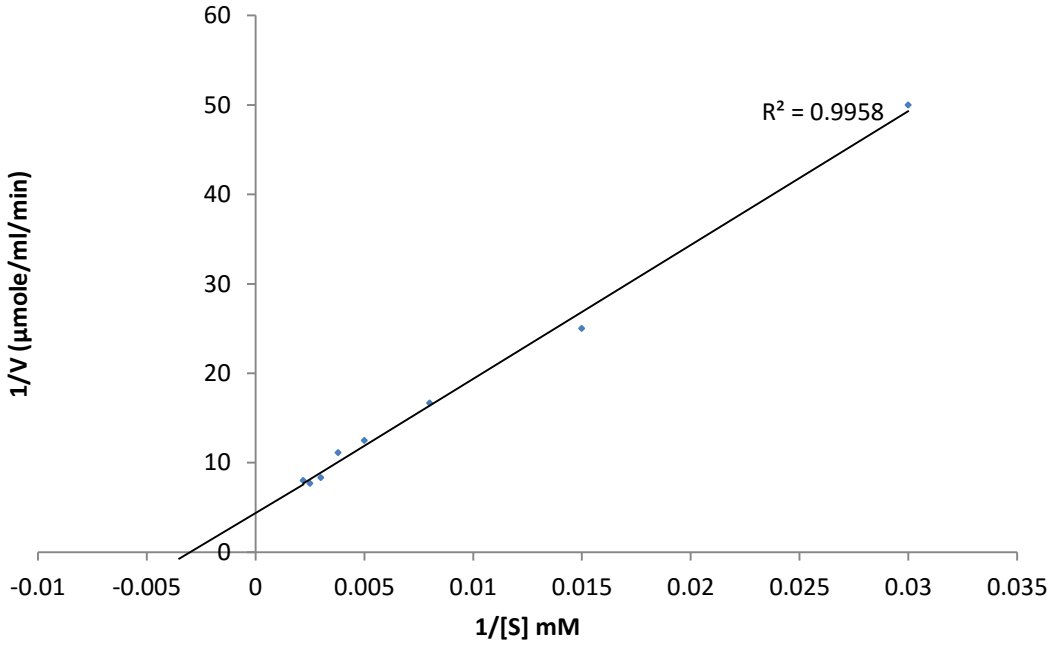


Figure 4.23: Double reciprocal plot of *A. nidulans* AN2227.2 β-glucosidase

The maximum velocity (Vmax) and the Michalis-Menten constant (Km) of *A. nidulans* AN2227.2 purified β-glucosidase for the substrate pNPG were 0.20 µmole/ml/min and 0.42 mM respectively.

Figure 4.24 and 4.25 (Appendix 21) shows the effect of substrate concentration on reaction velocity at constant enzyme concentration and the double reciprocal plot of *A. nidulans* AN1804.2 β-glucosidase activity. The enzyme activity increased with substrate concentration until all the enzyme was saturated with a substrate concentration of 100 mg/ml (Figure 4.24). The curve was characterized by a first order and zero order kinetics with a rectangular hyperbola shape.

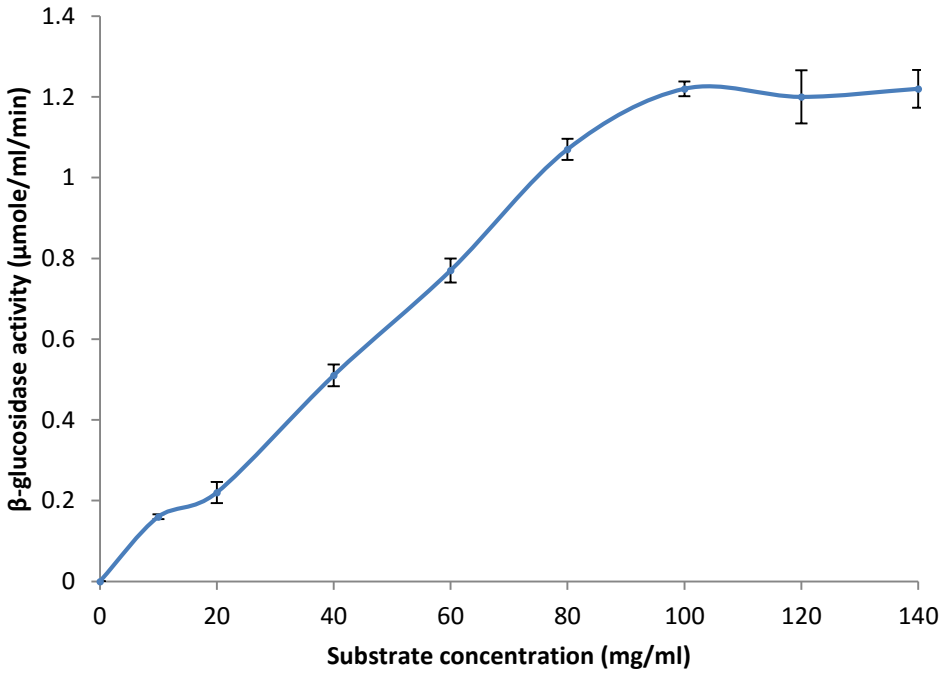


Figure 4.24: Effect of substrate concentration on the activity of *A. nidulans* AN1804.2 β-glucosidase

Error bars represent the mean ± SEM (n = 3)

The kinetic constants (Km and Vmax) calculated from Figure 4.25 for the purified *A. nidulans* AN1804.2 β-glucosidase were 0.59 mM and 2.04 µmole/ml/min respectively.

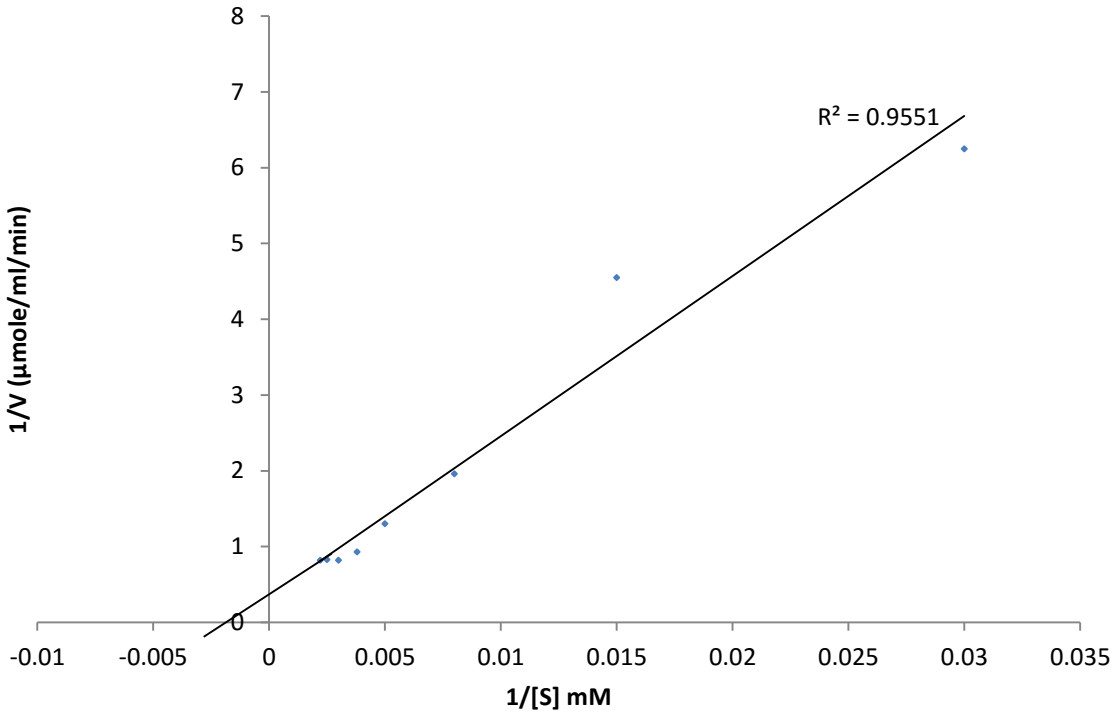


Figure 4.25: Double reciprocal plot of *A. nidulans* AN1804.2 β-glucosidase

4.3.6.7 Effect of Cations on β-glucosidase Activity

In this study, the effect of cations on the purified β-glucosidases was conducted using 0.02 M of $MgCl_2$, $CoCl_2$, $FeCl_3$, $CaCl_2$, $FeCl_2$ and $HgCl_2$. Figure 4.26 and 4.27 (Appendix 22) showed that both *A. nidulans* AN2227.2 and AN1804.2 β-glucosidases activity were similarly activated by $CoCl_2$, $FeCl_3$, $CaCl_2$, $FeCl_2$ and $ZnCl_2$ and deactivated by $HgCl_2$. $MgCl_2$ had minimal effect on both the enzyme activities.

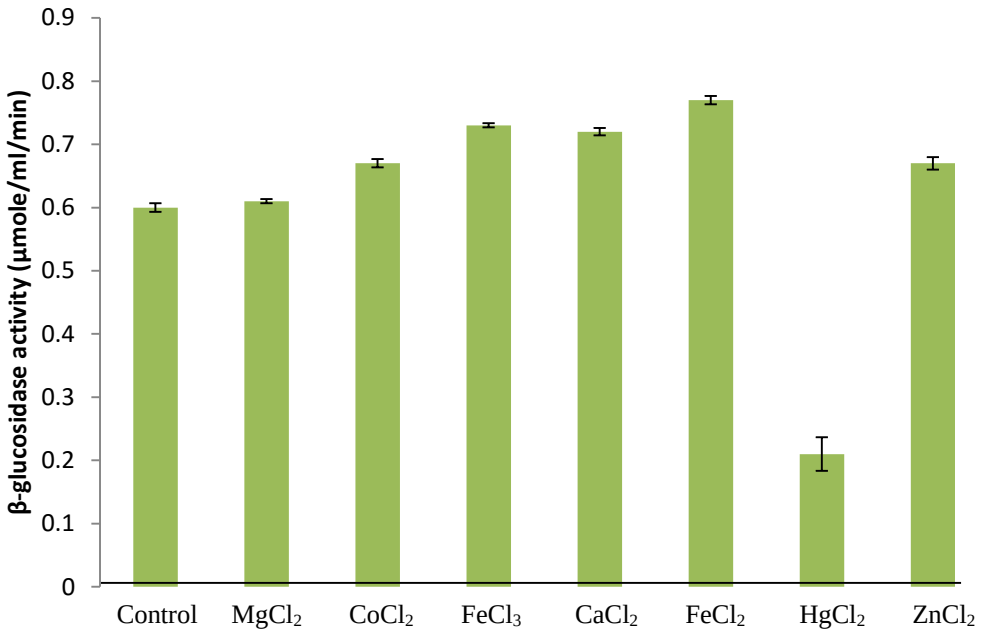


Figure 4.26: Effect of cations *A. nidulans* AN2227.2 β-glucosidase activity

Error bars represent the mean ± SEM (n = 3)

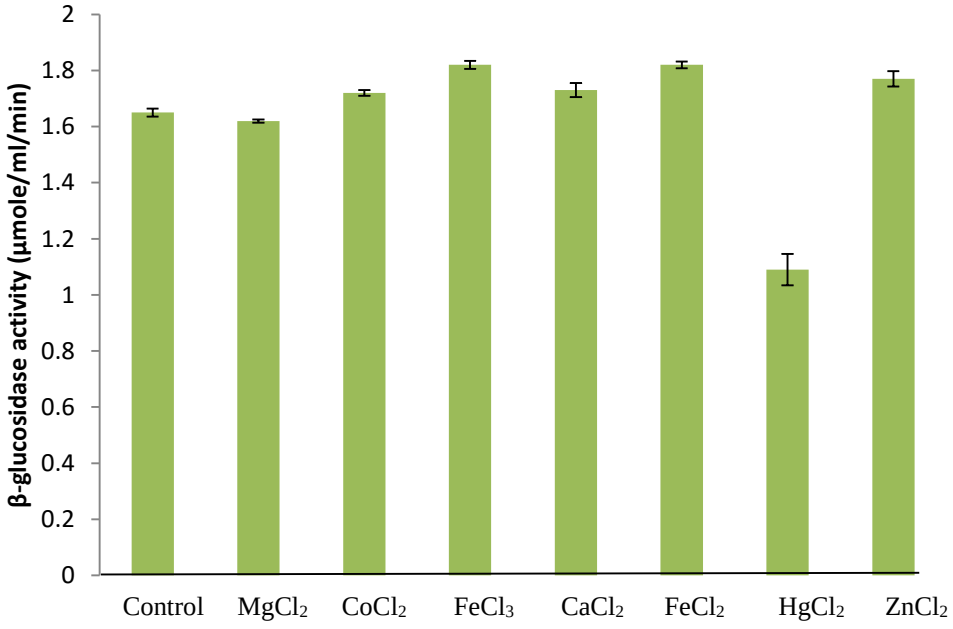


Figure 4.27: Effect of cations on *A. nidulans* AN1804.2 β-glucosidase activity

Error bars represent the mean ± SEM (n = 3)

4.4 Discussion

4.4.1 Screening of fungal and bacterial strains for cellulase enzymatic activity

Cellulolytic microorganisms can be isolated from soil, agricultural wastes and animal dung. For example, Saxena *et al*., (1993) isolated cellulose degrading bacteria from soil and termite guts. Teunissen *et al*., (1992) isolated an anaerobic fungus *Piromyces sp*. from the droppings of an elephant and found that the fungus was able to use cellobiose, cellulose, glucose, starch, wheat straw and wheat bran as carbon source.

The optimum conditions such as pH, temperature and effect of carbon sources for enzyme production were determined in these studies for a variety of microorganisms. Previous studies on enzyme production have shown that the initial pH of a medium has an influence on enzyme production and varies from species to species (Shaikh *et al*., 2013; Gautam *et al*., 2011; Chandel *et al*., 2013). The results from this study showed production of enzyme by the fungal strains screened to be at optimum between the acidic pH values of 4 – 6 with a concomitant decrease in activities towards alkaline pH values. The effect of pH on cellulase enzyme activity in this study agrees with the reports of Gautam *et al*., 2011 and Lynd *et al*., 2002 where fungal cellulases had optimum enzyme production within the acidic pH range. This report also supports the findings of Lee *et al*., (2002) and Chandel *et al*., (2013) who reported maximum cellulase production of the *Aspergillus* species within the acidic pH. It was also noted that bacterial enzyme activities were towards the alkaline pH with pH values of 8 - 9. While Song *et al*., (1985) also observed optimal cellulase production at pH 9.0 by *Clostridium acetobutylium*, most microorganisms are documented to grow and have cellulolytic activity within a wide pH range (Immanuel *et al*., 2006).

Temperature plays an important role in expressing the activity of biological system and has great influence on the production of end product (Chandel *et al*., 2013). Figure 4.5A and 4.5B showed optimum temperature for some selected fungal cellulase hydrolysis to be

between 40 ^{o}C and 45 ^{o}C. However, optimal enzyme activity for the bacterial strains was found to be between 40 ^{o}C to 50 ^{o}C. Previous research studies (Murao *et al*., 1988; Lu *et al*., 2003) have reported different optimum temperatures for cellulase enzyme production suggesting that maximum/optimal temperature for cellulase production depends on the strain of microorganism. The fungal and bacterial cellulase optimum temperature of 40 ^{o}C and 50 ^{o}C in this study tend to agree with the result of Shaikh *et al*., (2013) where the optimum activity of CDB30 cellulase enzyme from *Bacillus* species was reported to be highest at 50^{o}C. Pardo and Forchiassin (1999) also reported the optimal temperature for the activity of a cellulase system from *Nectria catalinensis* to range between 50 ^{o}C to 55^{o}C. An optimum temperature of 40^{o}C for cellulase produced by *A. niger* has also been reported by Gautam *et al.*, (2011).

Carbon sources play an important role in the growth of microorganisms and in the synthesis of cellulase enzyme. The carbon sources used in this study included glucose, CMC, cellobiose and cellulose. All the strains investigated had a luxuriant growth on glucose supplemented agar plates. This is because glucose is a primary carbon source for ATP production which is cheap, readily available and also rapidly metabolizable carbon source (Burgess, 2011). The cultivation of *A. niger* 320 and *A. niger* 321 on cellobiose and CMC supplemented plates resulted in little growth on cellobiose supplemented plates and no growth on CMC supplemented plates, even though they had a very good growth from initial studies where a small concentration of glucose was added in the media preparation. One explanation could be that in the earlier studies, the addition of glucose acted as an inducer for growth. It has been reported that glucose is suitable for optimum growth and cellulase production (Ramana *et al*., 2000). Glucose repression will usually ensure repression of enzymes for utilization of other carbon sources until the glucose is used up (Gorke and Stulke, 2008). Onyike *et al*., (2008) also reported the addition of xylose as an inducer for the production of cellulase enzyme from *A. niger*. The growth of *D. arenaria*

F200, F207, F.208; *A. nidulans* L.19 and POL.1 on cellulose (which may contain crystalline and amorphous cellulose) is an indication of cellulolysis. In this study, *D. arenaria* F207, *A. niger* F212, *A. nidulans* L19, *A. nidulans* L20, *A. nidulans* POL1 and *A. nidulans* GO281 had good growth in all the carbon sources used, indicating they will be good cellulase producers. The four *A. nidulans* isolates used in this study showed variation in their capacity to produce cellulases to degrade cellulose. These differences could be as a result of their genetic make-up, or may be due to the medium not being optimally supplemented to suit auxotrophic mutations like Para-aminobenzoic Acid (*paba*).

PABA is involved in primary metabolism and antibiotic biosynthesis. For instance, in *Streptomyces sp*. FR-008, a gene *pabC-1* putatively a fold-type IV pyridoxal 5'-phosphate (PLP)-dependent enzyme was found within the antibiotic FR-008/candicidin biosynthetic gene cluster, whose inactivation reduced the production of antibiotic FR-008 to about 20 % (Zhang *et al*., 2009). Several other studies have reported abnormal gene expression levels ascribed to suboptimal culture conditions (Gorgens *et al*., 2005; Zhang *et al*., 2009). Gorgens *et al*., (2005) supplemented a medium with amino acids to improve heterologous xylanase production by *Saccharomyces cerevisiae*. The workers results showed that the addition of a balanced mixture of the preferred amino acids, alanine, arginine, asparagine, glutamic acid, glutamine and glycine improved xylanase production. The genome of *Aspergillus nidulans* has already been sequenced by Galagan *et al*., (2005), therefore, studies involving the development of a gene targeting system in *Aspergillus nidulans* should facilitate the efficient production of β-glucosidases.

4.4.2 Small Scale Shake Flask Fermentation of *P. pastoris* clones AN2612.2, AN0712.2, AN1551.2, AN2227.2 and AN1804.2

Five *Pichia* clones carrying *A. nidulans* β-glucosidase gene (with accession No.: AN2227.2, AN2612.2, AN0712.2, AN1551.2 and AN1804.2) in pPICZ vectors that exhibit satisfactory levels of expression of recombinant β-glucosidase (Bauer *et al*., 2006) were used in this study for the expression of β-glucosidase enzyme. The experimental data from

BMMY culture media indicated an increase β-glucosidase enzyme hydrolysis of carboxymethyl cellulose (CMC) as the period of incubation increased. The trend was similar with all the *Pichia* clones with the exception of the wild-type *P. pastoris* 323 strains which had high cell growth media but no enzyme hydrolysis activities on CMC supplemented agar plates (Figure 4.7f ii). The *P. pastoris* 323 was not expected to have hydrolytic activity since it is not carrying any cellulolytic enzyme gene.

BMMY media used for enzyme production indicated effectiveness in β-glucosidase production. As the cells grow, the growth pattern showed a growth curve with three distinct regions for all the cells, that is lag phase, exponential phase and stationary phase. All the cell growth curves were characterized by decrease in cell growth during initial growth phases. Eighty percent (80%) of the β-glucosidase enzyme activities of the *Pichia* clones had maximum cell growth at 96 hours while only 10% had maximum growth at the 72 hours. Enzyme expression on BMMY medium started after 24 hours of inoculation with 80% of the *Pichia* clones reaching maximum enzyme production level after 72 hours of incubation. The decrease in β-glucosidase activity after the 72 hours could be as a result of the depletion of nutrients and/or the production of other by-products in the fermentation medium.

At the same incubation period of 72 hours, *P. pastoris* clones with accession number AN1804.2 recorded the highest activity (Figure 4.7h). *P. pastoris* clones with accession number AN0712.2 recorded the lowest enzymatic activity after 96 hours of incubation period.

Recently, many proteins have been genetically engineered and produced recombinantly in *P. pastoris*. Methylotrophic *P. pastoris* is a good choice for protein production because it is faster, easier to manipulate genetically, and has the ability to form post translational modification whilst being less expensive to use than other eukaryotic expression systems generally gives higher expression levels (Aoki *et al*., 2003; Invitrogen, 2010). Cells'

growth was characterized by decrease in cell growth during initial growth phases (Figure 4.7) which could be as a result of substrate inhibition which occurs while substrate concentration is high (Shuler and Kargi, 2002).

The time course of β-glucosidase production from engineered *P. pastoris*, yielded β-glucosidase enzyme after 24 hours of cultivation on BMMY medium (Appendix 26). Protein expression in *Pichia pastoris* provides the chance to produce large quantities of recombinant protein in a rapid and easy to use expression system. Being a single-celled microorganism and a eukaryote, *Pichia pastoris* is able to do many of the post-translational modifications performed by higher eukaryotic cells and the obtained engineered proteins undergo proteolytic processing, protein folding, glycosylation and disulphide bond formation (Weidner *et al*., 2010; Cereghino and Cregg, 2000). Although the bacterial expression systems, such as *E. coli*, have long been the favourite expression system for recombinant protein production, this system has some native defects. Being a prokaryotic based system, heterologous eukaryotic proteins expressed are not correctly modified, proteins are not correctly folded and tend to aggregate into inclusion bodies; insoluble and inactive proteins are co-produced due to codon bias, protein folding, glycosylation, phosphorylation, mRNA stability and promoter strength (Khow and Suntrarachun, 2012; Rosano and Ceccarelli, 2014). Relative to bacterial expression systems, the chances of recovering a eukaryotic foreign protein in its biologically active form are significantly higher with *Pichia pastoris* (Cereghino *et al*., 2001). Another advantage of the *Pichia pastoris* system is that it contains the AOX1 promoter for tightly regulated, methanol-induced expression of genes of interest and the α-factor secretion signal for secretion of recombinant protein (Weidner *et al*., 2010).

One important feature of AOX1 promoter is its ability to be switched on and off by changing carbon sources (Kupcsulik and Sevella, 2004). The switching between repressive glycerol to methanol carbon source could be responsible for the delay in extracellular

protein production on BMMY medium. Accumulation of toxic by-products such as H_2O_2 that is associated with methanol metabolism (Van der Klei *et al*., 2006) can affect the expression of protein. AOX promoters which are maximally induced at low methanol concentrations are also highly repressed in the presence of non-limiting concentrations of glucose (Hartner and Glieder, 2006; Promdonkoy *et al*., 2014). Rumjantsev *et al*., (2014) also investigated the effect of nitrogen source on methanol utilization in *Pichia pastoris*. Their results showed that expression levels of main genes involved in methanol utilization are maximal when ammonium sulphate, glutamine and glutamic acid are used as nitrogen sources, while expression is decreased in media with poor nitrogen sources such as proline or urea. Chen *et al*., (2015) used *Pichia pastoris* in the expression of β-mannanase from *Aspergillus niger* GIM3.452. In order to increase the secretion of β-mannanase, they constructed double recombinant yeast and made it coexpress protein disulphide isomerase. When produced under optimized conditions of methanol concentration and induction time, the β-mannanase activity increased from 40 U/mL to 222.8 U/mL. Several other studies have also reported high expression of protein levels using *Pichia pastoris* (Cereghino *et al*., 2002; Chen *et al*., 2007). Liu *et al*., (2012) also expressed a thermostable β-glucosidase (nBgl3) from *Aspergillus fumigatus* Z5 using *Pichia pastoris* X33. The activity of the purified nBgl3 showed optimal activity at pH 6.0 and 60 ºC.

Bauer *et al*., (2006) reported the production of a set of 74 plant cell wall hydrolytic enzymes from *A. nidulans* (72 enzymes) five of which are *Pichia* clones carrying *A. nidulans* β-glucosidase genes that are investigated in this study. Their experimental data show that clones with accession no AN0712.2 and AN1804.2 were active on pNP-β-glucoside substrate while clone with the accession no AN1551.2 is not active on the same substrate. Clones with accession AN2227.2 and AN2612.2 were not characterised for substrate activity in their studies. This gives an opportunity for the clones to be further exploited for their β-glucosidase activity in the future. In Chapter 3, the nature of the five

Pichia pastoris β-glucosidase clones from Bauer *et al*., (2006) were described using multiple sequence alignment (Section 3.3.5; Appendix 38) and phylogenetic tree (Section 3.3.5; Figure 3.4). Multiple sequence alignment revealed AN1551.2 protein to have poor alignment with AnBg11 protein (Lima *et al*., 2013) with only one active site residue (Appendix 38) and also forming a clade with proteins of bacterial origin (Figure 3.4). Meanwhile, Bauer *et al*., (2006) had reported that AN1551.2 protein is not active on pNP-β-glucoside substrate raising a concern whether AN1551.2 is actually a β-glucosidase. AN1804.2 protein had all the ten β-glucosidase active site residues when compared to AnBg11 protein (Lima *et al*., 2013) but lacked some of the *Aspergillus* amino acid insertions (Figure 3.2). The protein is also from the same clade as proteins of bacterial origin (Figure 3.4) thereby suggesting that AN1804.2 β-glucosidase is a potential HGT candidate.

4.4.3 Small scale expression of the recombinant *P. pastoris* strain β-glucosidase

In this study, β-glucosidases were purified from *A. nidulans* AN2227.2 and AN1804.2 clones expressed in *Pichia pastoris* (Bauer *et al*., 2006) using Buffered Methanol Complex Medium (BMMY). The expression of the enzyme was monitored by screening its activity. Figure 4.8 shows the time course for crude β-glucosidase production by *A. nidulans* AN2227.2 and AN1804.2 while Figure 4.9 shows the time course for crude β-glucosidase activity by *A. nidulans* AN2227.2 and AN1804.2. Although the crude β-glucosidase enzymes produced by *A. nidulans* AN2227.2 and AN1804.2 were found to reach optimum enzyme expression and activity of 0.52 μmole/ml/min and 1.78 μmole/ml/min respectively (Figure 4.8 and 4.9), a lag period was observed before the enzyme activity reached the peak value on the second day following methanol induction. The lag period suggested that there was adsorption of the methanol and as the hydrolysis proceeded, a portion of the adsorbed enzymes was gradually released into the reaction supernatant as suggested by *Lee et al*., (1994). β-glucosidases from *A. nidulans* AN2227.2 and AN1804.2 were successfully

expressed and the enzymes were used for biochemical characterization using pNPG as a substrate.

A purification of 1.02 fold was achieved for *A. nidulans* AN2227.2 β-glucosidase while a purification of 1.17 fold was achieved for *A. nidulans* AN1804.2 β-glucosidase after ammonium sulphate precipitation. This result is expected because ammonium sulphate has been reported to be the best protein precipitant without having effect on the biological activity of the enzyme (Onyike *et al*., 2008). Ammonium sulphate also has high solubility characteristics and changes of temperature have little or no effect on its solubility (Dixon and Webb, 1964). Dialysis produced a purification of 1.88 and 1.72 fold for *A. nidulans* AN2227.2 and AN1804.2 β-glucosidases respectively. The increase in purification is due to desalting and the removal of low molecular weight compound.

4.4.3.1 Anion-exchange chromatography

The extracellular β-glucosidases from *A. nidulans* AN2227.2 and AN1804.2 were purified by ion exchange chromatographuy using DEAE-sephadex A-50. The anion exchange chromatography separation of the enzymes by elution on DEAE-sephadex A-50 columns with linear gradient NaCl (0.05 M – 1.0 M) showed a single broad peak for both AN2227.2 and AN1804.2 β-glucosidases (Figure 4.10 and 4.11) suggesting the presence of a single active protein in the eluate. The purified enzyme preparation produced the final purification of 2.58 fold with 16.67 % recovery of enzyme activity for *A. nidulans* AN2227.2 β-glucosidase, and a purification of 2.25 fold with 33.33 % recovery of enzyme activity for *A. nidulans* AN1804.2 β-glucosidase. Kaur *et al*., (2007) reported 4.06 fold purification with 15.89 % recovery activity of β-glucosidase isolated from *Melanocarpus sp* MTCC 3922. The difference in the purification fold and percentage yield of β-glucosidase activity in this work and that reported by Kaur *et al*., (2007) is probably due to the difference in the source of the enzymes. The overall specific activity for AN2227.2 and

AN1804.2 β-glucosidases was improved from 2.13 and 1.81 to 5.50 and 4.08 respectively. These results indicated that sephadex A-50 column chromatography yielded effectively pure enzyme.

Judged by the SDS-PAGE data (Figure 4.12 and 4.13), the purified β-glucosidases from *A. nidulans* AN2227.2 and AN1804.2 were homogeneous. The molecular weight of the AN2227.2 and AN1804.2 β-glucosidases estimated by SDS-PAGE were 48 and 100 kDa respectively suggesting that the enzymes are monomers. Lima *et al*., (2013) purified a monomeric β-glucosidase from *A. niger* (AnBgl1) with an apparent molecular weigth of 116 kDa. The molecular weight of 100 kDa for *A. nidulans* AN1804.2 β-glucosidase correlate with 102 kDA molecular weight reported by Kaur *et al*., (2007) for β-glucosidase from *Melanocarpus* sp MTCC 3922, and the molecular weight of 90 – 91 kDa is also reported (Meko'o *et al*., 2012; Liu *et al*., 2012) for recombinant β-glucosidase from *Pichia pastoris*. Bai *et al*., (2013) also reported a molecular weight of 126 kDa for β-glucosidase isolated from *Penicillium simplicissimum*-11. Data from this study show the molecular weight of AN1804.2 β-glucosidase to be twice the molecular weight of AN2227.2, suggesting that AN1804.2 may have accesory proteins attached to it or that it is glycosylated. Though some β-glucosidases have a simple monomeric structures with around 35 kDa molecular weight, others have dimeric structure with molecular weight of 146 kDa or even trimeric structures with over 450 kDa (Mehdi *et al*., 2009).

4.4.3.2 Effect of pH and temperature on β-glucosidase activity

The maximum activities for the *A. nidulans* AN2227.2 and AN1804.2 β-glucosidases were observed at pH 6.0 and 5.5 respectively. Both enzymes had a broad acidic pH range and were not active in the extreme alkaline pH. The optimal pH of 6.0 for AN2227.2 is similar to pH 6.0 reported for AN1804.2 β-glucosidase (Bauer *et al*., 2006; Liu *et al*., 2012) while the optimal pH of 5.5 for AN1804.2 is near pH 6.0 reported for same enzyme by Bauer *et*

al., (2006). A pH of 4.4 – 5.2 was also reported for β-glucosidases isolated from *Penicillium simplicissimumh*-11 (Bai *et al*., 2013). The pH optimum for AN2227.2 and AN1804.2 reported in this work is also in agreement with those reported for most fungal sources which are near pH 6.0 (Wei *et al*., 1996; Bhat *et al*, 1993; Bauer *et al*, 2006; Kaur *et al*., 2007). Reports have also shown that maximum activity at an acidic pH is a common property of cellulases from fungal sources (Alam *et al*., 2004).

The effect of temperature on the activity of the purified β-glucosidases on pNPG was also analyzed. The maximum activity for the *A. nidulans* AN2227.2 and AN1804.2 β-glucosidases were observed at 40 and 50 ^{o}C respectively. The lower value of 40 ^{o}C for AN2227.2 β-glucosidase agreed with the value of of 40 ^{o}C reported for β-glucosidase from thermophilic fungus *Humicola insolens* (Moreira *et al*., 2010) but lower than that reported for most β-glucosidases; while the value of 50 ^{o}C for AN1804.2 β-glucosidase falls within the range for most β-glucosidases (Liu *et al*., 2012; Bai *et al*., 2013; Kaur *et al*., 2007). Both optimum temperatures were lower than that reported for β-glucosidase from *Aspergillus niger* KCCM 11239 (Chang *et al*., 2012) with an optimum temperature of 70 ^{o}C. The optimum temperature value of 50 ^{o}C for AN1804.2 β-glucosidase obtained in this work is in agreement with that reported by Bauer *et al*., (2006) for the same enzyme. The thermostability at 50 ^{o}C for AN1804.2 β-glucosidase reported in this work is within the range reported by Kaur *et al*., (2007). However, the most striking characteristics of these enzymes, particularly AN1804.2, are the dramatically broad pH and temperature profiles.

4.4.3.3 Kinetic Constants (K_m and V_{max})

The Km is a means of characterizing an enzyme's affinity for a substrate. A low Km value means that the enzyme has a high affinity for the substrate, as a little substrate is enough to run the reaction at half its speed (Kaur *et al*, 2007). Figure 4.24 revealed that β-glucosidase from *A. nidulans* AN2227.2 has a lower value of Km 0.42 mM for pNPG than β-

glucosidase from *A. nidulans* AN1804.2 which has a Km value of 0.59 mM indicating higher affinity of the enzyme for pNPG substrate. The Km values for both AN2227.2 and AN1804.2 β-glucosidases obtained in this study are higher values for pNPG than the Km of 0.117 mM, 0116 mM, 0.057 mM and 0.3 mM for β-glucosidase from *Fomitopsis palustris*, *Humicola insolens*, *Peniclillium funiculosum* NCL1 and *Phoma* sp KCTC11825BP respectively (Yoon *et al*., 2008; Choi *et al*., 2011; Ramani *et al*., 2012; Bhatti *et al.*, 2013) and lower than the Km of 3.11 mM, 1.9 mM, 2.5 mM and 2.67 mM for β-glucosidases isolated from *Aspergillus niger* NRRL 599, *Aspergillus saccharolyticus*, *Aspergillus terreus* NRRL 265 and *Trichoderma koningii* AS3.2774 respectively (Lin *et al*., 1999; Elshafei *et al*., 2011; Zahoor *et al*., 2011; Sørensen *et al*., 2012).

4.4.3.4 Effect of Cations on β-glucosidase Activity

The effect of cations on the purified *A. nidulans* AN2227.2 and AN1804.2 β-glucosidases enzyme as indicated on Figure 4.26 and 4.27 showed that the enzyme activities are slightly enhanced in the presence of almost all the cations tested. Only $HgCl_2$ caused 25.93% and 39.64% loss in activity of AN2227.2 and AN1804.2 β-glucosidases respectively, indicating inhibition. These studies indicate that β-glucosidases may not be metalloproteins and therefore do not require metal ions for optimum activity (Pei *et al*., 2012). The slight activation by $CoCl_2$, $FeCl_3$, $CaCl_2$, $FeCl_2$ and $ZnCl_2$ may be explained by stabilization of the enzyme. This result is in agreement with the report of Meko'o *et al*., (2012), where they reported that 1% EDTA (a chelating agent) did not affect the activity of β-glucosidases expressed from *P. pastoris*, indicating that β-glucosidases are not metalloproteins. The sensitivity of β-glucosidase enzymes to mercuric chloride ($HgCl_2$) suggests that SH-groups may be involved in the enzyme catalyzed reaction mechanism. This is because heavy metals like $HgCl_2$ would selectively react with –SH residues of the enzyme thereby inactivating it (Dixon and Webb, 1964; Onyke *et al*., 2008).

4.5 Conclusion

The present study was carried out to develop a rapid plate-based assay for cellulase activity and characterize *Pichia* clones carrying *A. nidulans* β-glucosidases gene. The plate assay was rapid and lots of samples were analysed within a short time. *B. subtilis*, *A. niger* F320 and *A. niger* F321 appeared to be the most efficient cellulase producers by their ability to degrade CMC. The *Pichia pastoris* β-glucosidase clones indicated that AN1804.2 is a very good producer of β-glucosidase on BMMY media. The characteristics of two *A. nidulans* (AN2227.2 and AN1804.2) recombinant β-glucosidases expressed from *P. pastoris* were also described, with high-level expression of the AN1804.2 β-glucosidase. The study suggests both AN2227.2 and AN1804.2 protein to be present in monomeric form with AN1804.2 enzyme in particular having a good pH and temperature stability making it a good candidate in cellulose hydrolysis.

Chapter 5

Production and degradation of bacterial cellulose, cloning and characterization of novel β-glucosidases from *Gluconacetobacter xylinus* (*G. xylinus*)

5.1 Introduction

Cellulose is the most abundant macromolecule on earth and is one of the major components of the dry mass in materials such as cotton (90%) and wood (50%), (Perez-Fuentes *et al*., 2014; Hon, 1994; Keshk, 2014). The biosynthesis of cellulose is not exclusively found in plants alone but can be found in a variety of microorganisms such as algae, bacteria and oomycetes (Brown, 2004; Blum *et al*., 2012). Bacterial cellulose (BC), also known as microbial cellulose and bacterial nanocellulose, is produced by bacteria such as *G. xylinus* (originally named *Acetobacter xylinum*), *Achromobacter*, *Rhizobium*, *Agrobacterium*, and *Sarcina* (Jonas and Farah, 1998; Matthysse *et al*., 1995). *G. xylinus* acquires glucose and other organic substrates converting them into pure cellulose with unique structural and mechanical properties which are highly pure because of a lack of contaminating lignin and hemicellulose as compared to plant cellulose (Son *et al*., 2001; Gayathry and Gopalaswamy, 2014). These properties make BC advantageous for biotechnology applications, for example in the production of biodegradable polymers and third generation biofuels. BC is secreted as an extracellular insoluble biofilm and forms a gelatinous mat on the air liquid interface due to its comparatively low density to water. Biosynthesis of BC requires a carbon source that is converted into Glucose-6-Phosphate, a metabolic intermediate for the formation of precursor substrate uridine diphosphoglucose (UDPGlc), which acts as a D-glucose donor to cellulose synthase facilitating the polymerization of a β (1→4) D-glucan chain (Ross *et al*., 1991). Many other carbon sources that are alternative to glucose have been reported in the production of BC by *G. xylinus* (Keshk, 2014; Mikkelsen *et al*., 2009; Sherif and Kazuhiko, 2005).

The synthesis of cellulose in higher plants is mediated by cellulose synthase complexes (CSCs) localized on the plasma membrane (Vain *et al*., 2014; Song *et al*., 2010). Cellulase synthase (CS) complexes are encoded by the CS operon which codes for four different

bacterial cellulose biosynthesis (bcs) subunits, *bcsA*, *bcsB*, *bcsC* and *bcsD* in *G. xylinus* (Figure 5.1). The *bcsA* product functions in the acceleration of cellulose synthesis by combining with cyclic-di-GMP, while the *bcsB* product is involved in inner membrane attachment and the catalysing unit (Wong *et al*., 1990). Though the primary functions of *bcsC* and *bcsD* are still not known, they are thought to be involved in the aggregation of each cellulose chain (Krystynowicz *et al*., 2005; Amano *et al*., 2005). Through extensive inter- and intra-fibrillar hydrogen bonding of the hydroxyl and ester groups, the sub-elementary fibre aggregates and crystallizes to form a micro-fibril (Keshk, 2014; Ross *et al*., 1991). Depending on the formation and organization of the chain, cellulose is assembled in a parallel fashion known as type I cellulose which has high crystalline structural morphology with increased strength and stability, or into type II cellulose which is composed of antiparallel chains and results in an amorphous structure (Sarkar and Perez, 2012). Though many other microbes produce cellulose, those celluloses produced by *G. xylinus* have better quality and are more stable, and are also produced in a larger quantity (Ross *et al*., 1991). BC therefore provides an attractive basis for polymer-based structures such as carrier bags or drinks bottles that could be recycled after use. If bacterial cellulose can be efficiently produced in commercial quantity and hydrolysed to fermentable sugars by enzymatic hydrolytic processes, it may then compete favourably with fossil fuel to meet future energy needs.

Ha *et al*., (2008) used the waste stream from beer culture fermentation instead of using the chemically defined medium to produce bacterial cellulose by a static cultivation. Tsouko *et al*., (2015) also demonstrated that by-product streams from the biodiesel industry and waste streams from confectionary industries could be used as the sole sources of nutrients for the production of bacterial cellulose. The production of bacterial cellulose using industrial waste could lead to cost competitiveness of industrial bacterial cellulose production.

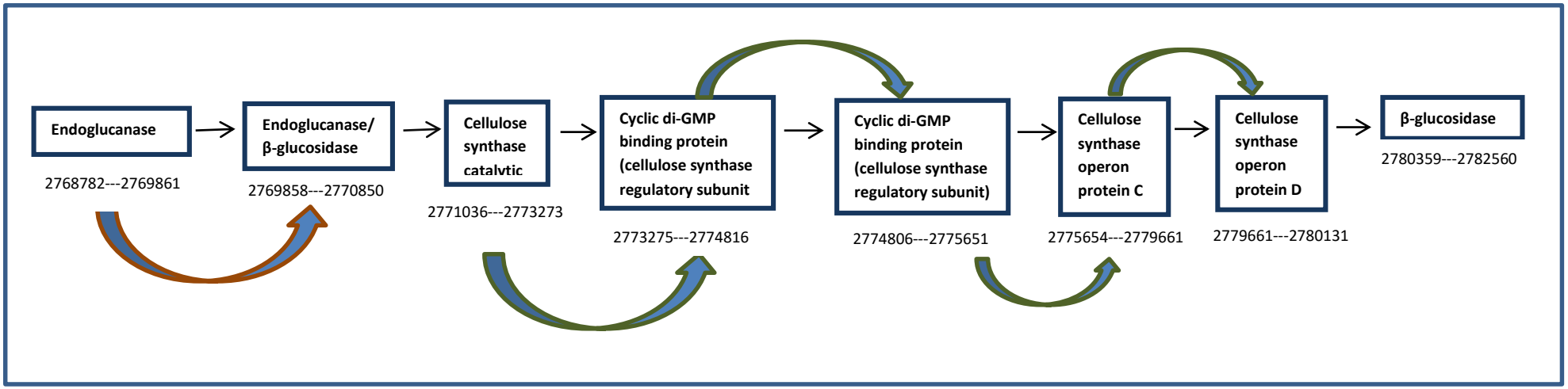


Figure 5.1: Schematic representation of *G. xylinus* NBRC 3288 cellulase complex. Diagram derived from annotation for bases 2768782 – 2782560 (NCBI GenBank Accession number AP012159.1). Figures in the diagram show relative positions of the individual genes while the curved up and curved down arrows indicate the connection between the gene sequences

Intriguingly, *G. xylinus* strains which are known to be efficient producers of bacterial cellulose can also produce β-glucosidase (Tajima *et al*., 2001). Even though β-glucosidase does not act on cellulose directly, it is of great importance by eliminating cellobiose inhibition on exoglucanase and endoglucanase which makes it a good candidate in the hydrolysis of cellulose for industrial application. Although these enzymes are known for their hydrolytic capabilities, they have been reported to play a role in the enhancement and production of BC (Standal *et al*., 1994; Tonouchi *et al*., 1995; Koo *et al*., 1998). β-glucosidases have the capability to catalyse and polymerise cellulose chains (Kono *et al*., 1999) and the encoding gene is thought to be located downstream of the cellulose synthase (CS) operon in *G. xylinus* (Tonouchi *et al*, 1997; Tajima *et al*, 2001). Figure 5.1 summarises the arrangement of genes in the *G. xylinus* NBRC 3288 cellulase complex. The schematic representation of the cellulase complex shows the cellulose synthase complex to contain a gene encoding a cellulose synthase catalytic subunit, a cyclic di-GMP binding protein (cellulose synthase regulatory subunit), a cellulose synthase operon protein C and a cellulose synthase operon protein D. These are flanked by genes encoding endoglucanases, endoglucanases/β-glucosidases and β-glucosidases.

There are several software applications such as BamView, LookSeq, MagicViewer and EagleView that have been developed for browsing, visualizing and interpreting of sequencing datasets (Carver *et al*., 2012). Artemis is a free genome browser and annotation software tool that is widely used in the annotation and viewing of sequence data. It is straightforward, suited for small genomes, requires no database other than a DNA sequence and individual sequence data can be analyzed with little or no formatting (Berriman and Rutherford, 2003). The use of Artemis in the annotation of cellulose synthase genes in this study helped with the study of relevant gene products that are encoded by the genomes.

This chapter is aimed at characterizing the production of bacterial cellulose (BC) from a *G. xylinus* strain and attempting the degradation of the BC produced, using a commercial fungal cellulase enzyme. The BC produced was characterised by scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, Differential scanning calorimeter (DSC), Thermogravimetric analysis (TGA) and Nuclear magnetic resonance (NMR). The study also attempted to clone and characterize β-glucosidases found in the cellulase gene region of *G. xylinus.* Finally, using Artemis to check the annotation of genes in the *G. xylinus* cellulase complex was essential.

5.2 Materials and methods

5.2.1 Microorganism

The *Gluconacetobacter xylinus* 639 (*G. xylinus*) isolate used in this study was obtained from the organisms collection centre of the Microbiology laboratory, University of Wolverhampton, Wolverhampton United Kingdom.

5.2.2 Culture medium and growth conditions for BC production

Hestrin and Schramm (1954) medium (HS medium) with initial pH adjusted to 6.0 using acetic acid was used to produce BC. HS medium was made up of (g/l): glucose – 20, bactopeptone – 5, yeast extract – 5, disodiumpohosphate – 2.7 and citric acid – 1.15. Inoculum was prepared by transferring two colonies of *Gluconacetobacter xylinus* from an HS agar plate into a 250 ml conical flask containing 100 ml sterile HS medium. The culture was incubated at 25^{o}C on a water rotary shaker (Model G25, S/No. 390534557 U/K) for 24 hours at 150 rpm. After 24 hours, the cellulose pellicle formed on the surface of the culture broth was vigorously shaken in order to remove active cells embedded in the cellulose membrane. 6 ml of the cell suspension was introduced into 94 ml of fresh HS medium. The culture was cultivated statically at 30^{o}C for 9 days. The synthesized cellulose was harvested and then purified by boiling in 1% NaOH for 2 hours and next it was thoroughly washed in tap water until the product was transparent while the second sample was washed with distilled water only. The samples were freeze dried for further analysis.

5.2.3 Genomic (g) DNA Extraction

G. xylinus genomic DNA was extracted using PureLink^{TM} Genomic DNA Mini kit (Catalog No. K1820-00) according to manufacturer's instructions. The extracted genomic DNA was used as a template for PCR reactions.

5.2.4 Primer design

Primers were designed based on the *Gluconacetobacter xylinus* β-glucosidase DNA sequence (Table 5.1) obtained from the genome database which is available on (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The coding sequence of *Gluconacetobacter xylinus* NBRC 3288 DNA, complete genome (Accession No. – AP012159.1) was chosen for this experiment, copied and pasted on NCBI (Primer design tool) to design the primer from protein-coding regions of the gene.

Table 5.1: List of specific primers designed for PCR amplification of *G. xylinus* 639 plasmid DNA (Sigma-Aldrich)

Serial No.	Sequence accession	Sequence (5' – 3')	Tm (°C)
1	AP012159.1 - F	TTATATTGCCCGTGGCGAGG	63
2	AP012159.1 - R	GGGGCCTGCATCCCTTATC	63

F: Forward primer, R: Reverse primer

5.2.5 Cellulase pretreatment of BC

Cellulase (1,4-(1,3:1,4)-β-D-glucanohydrolase) from *Aspergillus niger* (Sigma-Aldrich, Catalogue No. - C1184) was used in the hydrolysis of the BC. The BC was incubated overnight with 2 mg/ml cellulase enzyme at 37 °C. To inactivate the enzyme adsorbed to the BC, 1 M NaOH (pH 13.3) was used to treat the hydrolysed BC for 10 minutes. The enzymatically pre-treated BC was then thoroughly washed with distilled water and freeze dried for further analysis.

5.2.6 Characterization of bacterial cellulose (BC)

To study conformational characteristics, the bacterial cellulose (BC) polymer was characterized using microscopic and spectroscopic methods. The microscopy method used was Scanning Electron Microscopy (SEM)**.** The spectroscopy techniques used included Fourier Transform Infrared Spectroscopy (FTIR), X-Ray Diffraction (XRD), Nuclear Magnetic Resonance (NMR), Differential Scanning Calorimeter (DSC) and Thermogravimetric analysis or thermal gravimetric analysis (TGA).

5.2.6.1 Scanning Electron Microscopy (SEM)

The BC fibril (cellulase treated and untreated BC) was characterized using SEM (Zeiss Evo 50 fitted with an Oxford EDX, Zeiss, UK). Scanning electron microscopy reveals information about the BC chemical composition, crystalline structure and the external morphology of the sample. A thin layer of freeze dried BC was gold coated using an ion sputter-coater to improve the conductivity of the samples and the quality of the SEM images (emscope SC 500). The gold coated sample was viewed and the images were digitally captured.

The principle of SEM is that it uses electrons to form an image. SEM uses a focused beam of high energy electrons produced at the top of microscope to generate a variety of signals at the surface of solid specimens. The accelerated electrons dissipate their energy as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample (Bozzola and Russell, 1999). These signals including the secondary electrons produce SEM images.

5.2.6.2 Fourier Transform Infrared Spectroscopy (FTIR)

To study conformational characteristics of BC obtained from HS medium, polymer was analysed by FTIR spectrometer (Genesis II with DuraScope, Mattson Instruments UK) in a transmittance mode at wavelength ranging from 4000 cm^{-1} to 400 cm^{-1}. The FTIR provided

information on chemical structures and physical characteristics of the BC produced. BC samples were initially freeze dried and made into powder. A little quantity of the sample (or cellulase treated BC), just enough to cover the disc hole was placed on the disc. The disc was pressed and the scan of BC was operated on the PC which further generates the IR.

The working principle of FTIR is that FTIR involves the twisting, rotating, bending, and vibration of the chemical bonding. BC adsorbs infrared (IR) radiation from the photospectrometer and the extent of adsorption is determined by the atomic mass, length, strength and force constant of interatomic bonds in the structure of the BC (Lamberti, 2004). The multiplicity of vibrations occurring simultaneously produces a highly complex absorption spectrum, which is a unique characteristic of the functional groups comprising the molecule and also the configuration of the atoms. A detector monitors the wavelength range and transmits the signal to a computer which translates the signal into an absorption spectrum (Lamberti, 2004).

5.2.6.3 X-Ray Diffraction (XRD)

X-ray diffraction (XRD) is a rapid analytical technique that is used in the qualitative identification of crystalline phases by their diffraction pattern. In this study, XRD analysis was utilised to characterize the crystallinity index and the degree of polymerisation of the BC sample. This was performed using the Empyrean PANalytical diffractrometer (Philips model PW1770) at the University of Wolverhampton. BC was ground and homogenised before analysis. The sample was then placed in the sampling tray where the surface was smoothed to eliminate surface irregularities.

The principle of XRD is based on Bragg's law which states that $n\lambda=2d\sin\theta$.

Where:

n = difference in path length between adjacent x-ray beams (order of reflection)

λ = wavelength of the beams (incident X-rays) and is a known value

d = spacing between the crystal planes (inter-planar spacing) and

θ is the angle of scattering (incidence)

On the basis of Bragg's law and by measuring the angle (θ), wavelength (λ) can be determined and thus the chemical elements, if the lattice plane distance (d) is known or, if the wavelength (λ) is known, the lattice plane distance (d) and thus the crystalline structure can be determined (Lodeiro *et al*., 2009).

5.2.6.4 Differential Scanning Calorimeter (DSC)

Differential scanning calorimeter (DSC) is a thermo-analytical technique used to study what happens to polymers when they are heated, that is thermal transitions of a polymer. Two aluminium pans are used; the sample is placed in one pan while the second pan which serves as a reference pan is left empty. The two pans are heated at a specific rate, say 20 ^{o}C per minute. The principle underlying this technique is that when the sample undergoes a physical transformation such as phase transition, more or less heat will need to flow to it than the reference to maintain both at the same temperature. Whether less or more heat must flow to the sample depends on whether the process is exothermic or endothermic (Bhadeshia, 1974).

For untreated BC, approximately 8.2220 mg of BC from the compression molded products was loaded into aluminium pans. DSC measurements were taken with a TA-DSC 2010 apparatus (TA Instruments, Newcastle, DE, USA), under a nitrogen atmosphere (flow = 50mL/min). The instrument was calibrated with high purity indium. The melting temperature (T_m) was taken as the peak temperature maximum of melting endotherm. In this study, T_g was taken as the midpoint of the step-transition.

A first temperature a program was followed to record Tg and Tm. After heating to 200 ^{o}C at 20 ^{o}C/min in order to erase thermal histories, the BC samples were cooled down to -100 ^{o}C at a cooling rate of 30 ^{o}C/min. A second heating scan was conducted from -70 ^{o}C to 280 ^{o}C at 20 ^{o}C/min and two thermal transitions were recorded. The third heating scan was conducted again from -30 – 3500 ^{o}C at 20 ^{o}C/min and melting transition at 103.21 ^{o}C and Tg at 38 ^{o}C was recorded. For cellulase treated BC, approximately 14.2590 mg BC was used and the same procedure was carried out as described above with only first and second scan rounds using the same conditions.

5.2.6.5 Thermogravimetric analysis or thermal gravimetric analysis (TGA)

TGA is a thermo-analytical technique in which changes in weight are measured as a function of increasing temperatures. The basic principle of TGA is that as a sample is heated, its mass changes. This change can be used to determine the composition of a material or its thermal stability up to 1000 ^{o}C (Coats and Redfern, 1963).

To determine the thermal stability of untreated and cellulase treated BC, approximately 3.4830 mg of the untreated BC and 5.6700 mg of the cellulase treated BC were placed in aluminium pans. Raw and derivative weight data were used to determine decomposition, temperatures and associated weight losses. TGA analysis was performed with a TGA/DSC1 Mettler-Toledo thermal analyser at a heating rate of 10 ^{o}C/min in a stream of nitrogen (60 mL/min). The obtained TGA data were analysed using the Mettler-Toledo star system SW 9.30.

5.2.6.6 Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique that is used to determine the content and purity of a sample as well as its molecular structure. The principle of NMR spectroscopy is that many nuclei have spin and all nuclei

are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level. The interaction of nuclear magnetic moment (μn = (h/2n) Yn I) with an external magnetic field, B_o induces the splitting of the $2I + 1$ energy level of the nuclear spin I (Sanz and Serratosa, 1984).

Solid-state NMR spectra were obtained at the EPSRC UK, National solid-state NMR service at Durham. The solid state ^{13}C NMR spectra were measured on a Varian VNMRS 600 spectrometer. When untreated and cellulase treated BC samples were irradiated with a radio frequency, absorption of the resonant energy between adjacent energy levels occurs. The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy was emitted at the same frequency. The precise frequencies at which the spin-active BC resonates was picked up and displayed by the NMR spectrometer.

5.2.7 DNA isolation and PCR conditions

G. xylinus genomic DNA was extracted using PureLink™ Genomic DNA Mini kit (Catalog No. K1820-00) according to manufacturer's instructions. The extracted genomic DNA was used as a template for PCR reactions. Specific primers were designed based on *Gluconacetobacter xylinus* β-glucosidase DNA sequence (AP012159.1) obtained from a genome database which is available on (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The primer design for *G. xylinum* β-glucosidase was restricted to coding sequence (CDS) 2780359 --- 2782560 (Figure 5.1). The general conditions for Polymerase Chain Reactions (PCR) were carried out as described in section 6.2.5 outlined in Table 6.4. Purification of the amplified β-glucosidase gene fragment from the agarose gel was carried out using the QiagenMinElute® Gel Extraction Kit (2011) as described in section 6.2.7. The gel purified β-glucosidase DNA was re-amplified using the PCR reaction. Part of the PCR product was examined using agarose gel electrophoresis as described in section 6.2.6 while the other

part was purified using the Qiagen QIAquick^{R} PCR Purification Kit (2011) as described in section 6.2.8.

5.2.8 Cloning of *G. xylinus* β-glucosidase gene

Cloning of the β-glucosidase gene was carried out as described in section 6.2.9. Ligation and transformation reactions were as described in section 6.2.12 using pGEM-T for the TA ligation method and transformed into *E. coli* JM109 strains.

5.2.9 Plasmid purification of cloned fragments

To confirm that the β-glucosidase gene was successfully inserted in the pGEM –T cloning vector, colony PCR was carried out after plasmid DNA extraction and purification as described in section 6.2.16.

5.2.10 Sequencing of plasmid DNA

The cloned sequence from *G. xylinus* (AP012159.1) sample pDNA was sent to Source BioScience LifeSciences and Eurofins Genomics for sequencing using the same forward primers (5'-TTA TAT TGC CCG TGG CGA GG-3') as described in section 6.2.18. Sequencing of the *G. xylinus* (AP012159.1) β-glucosidase insert returned a 678 bp fragment. Sequence results from the companies were read using Chromas Lite 2.1.1–free Technelysium software for DNA sequencing (http://technelysium.com.au/?page_id=13) and analysed by BLAST. The sequences reads of appropriate quality obtained were compared against the non-redundant nucleotide sequence collections at Genbank using the web interface of NCBI-BLAST.

5.3 Results

In this study, BC was obtained as a thick layer from HS medium and the average wet yield of cellulose by *G. xylinus* was found to be 4.2 g/l with an average dry yield of 0.21 g/l. The BC samples used for analysis were harvested from a single flask of HS media containing multiple layers of BC (Figure 5.2). The multiple layers of BC were thought to be caused by the formation of a new pellicle due to the preceding pellicle sinking.

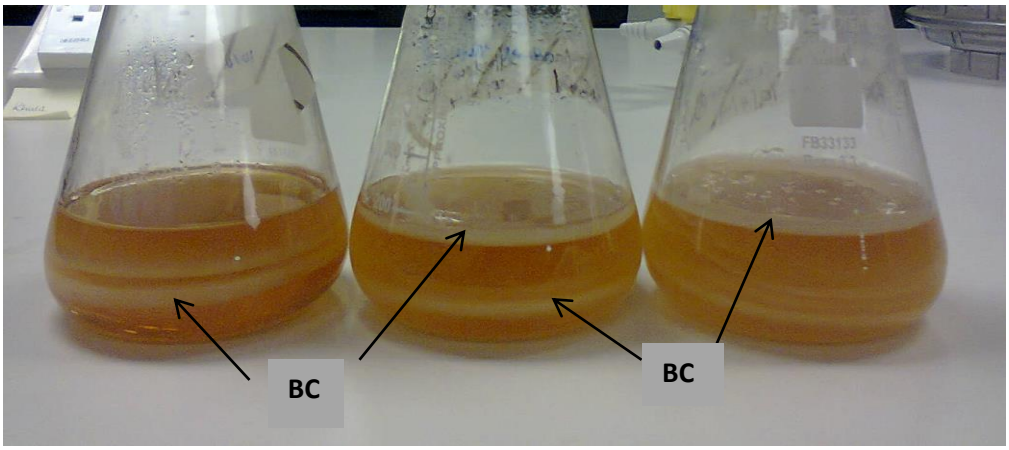


Figure 5.2: Layers of bacterial cellulose formed by *G. xylinus* 639 in triplicate after 8 hours in HS medium

This unique opportunity of formation of new pellicle due to preceding pellicle sinking eliminates much variability that can affect the quality of the BC thus allowing a better comparison among samples. All samples were similar in appearance and thickness. Samples were processed with distilled water (dH_2O), dH_2O + NaOH, cellulase pretreatment and were freeze dried before analysis.

5.3.1 Fourier Transform infrared spectroscopy (FTIR) Analysis

Table 5.2 is a summary table of corresponding wave numbers derived from the FTIR analysis associated with interpreted bonds and functional groups (Dai and Fan, 2010; Fan *et al*., 2012; Nam *et al*., 2011).

Table 5.2: Summary table of corresponding wave numbers derived from the FTIR analysis associated with interpreted bonds and functional groups (Dai and Fan, 2010; Fan *et al*., 2012; Nam *et al*., 2011).

Wavenumber Peaks (cm^{-1})	Type	Type of vibration	Functional group
3328	Broad	Stretching	-OH (H-bonded)
2950	Narrow	Stretching	C-H
1620	Narrow	Bending	-OH of absorbed water
1380		bending	Planar CH
1153		Stretching	Asymmetrical C-O-C
1020		vibration	C-C, C-OH, C-H ring and side group

Figure 5.3 shows the FTIR spectra in 4300 – 300 cm^{-1} region of BC and standard cellulose from Sigma-Aldrich. Both diffraction curves are of typical cellulose structure with a few exceptions of the BC spectra from the standard cellulose spectra. FTIR spectra in 1620 - 1380 cm^{-1} region show the band of the NaOH washed BC to be shifted. The bands at 37000 - 3000, 2970 – 2800, 1100 – 1073 and 1034 – 1023 cm^{-1} are similar.

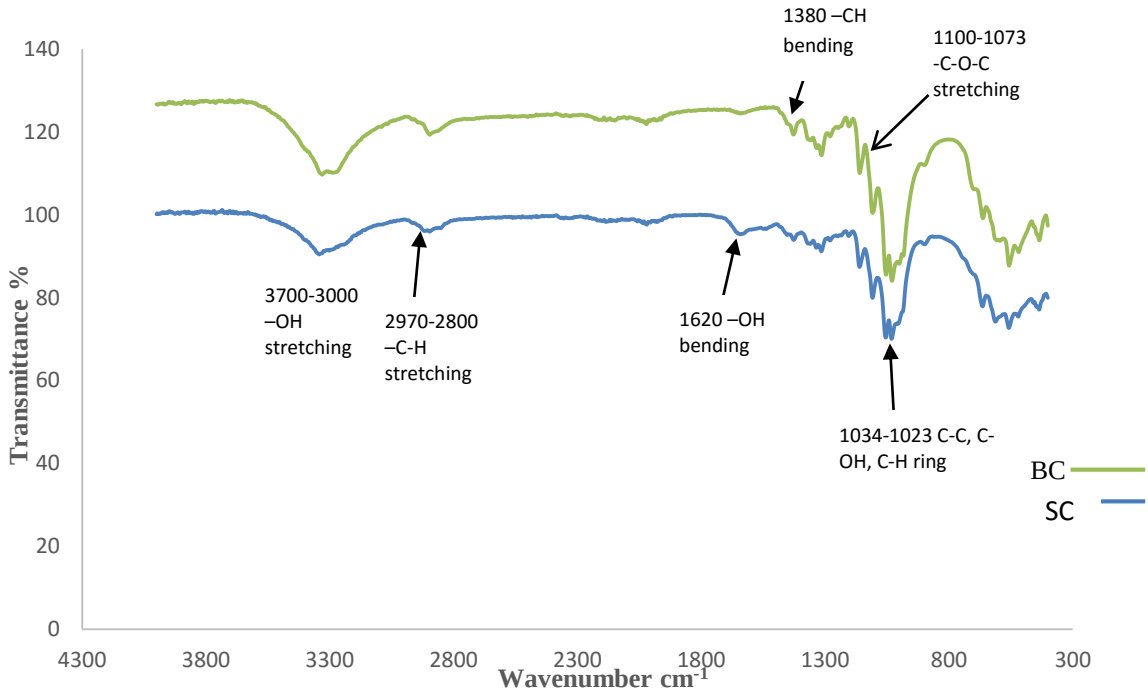


Figure 5.3: FTIR spectrum of freeze-dried BC from *G. xylinus* 639 and standard cellulose from Whatman. SC: Standard cellulose, BC: Bacteria cellulose

Figure 5.4 (A and B) show the FTIR spectra in the 3800 – 600 cm^{-1} region of BC and cellulase hydrolyzed BC which were employed to characterize the structure of the BC material before and after treatment with cellulase enzyme. The spectra shown in Figure 5.4 A show the absorption spectra band at 3338.41 and 2917.85 cm^{-1} for –OH stretching and –C-H stretching respectively. The absorption spectra band at 1015.65 cm^{-1} (Figure 5.4 B) is assigned as C-C, C-OH, C-H ring and C-O-C stretching at the β-(1→4)-glycosidic bond/linkage in cellulose (Nelson and O'Connor, 1964) while the absorption spectra band at 1424.13 cm^{-1} is assigned to the CH_2 scissoring motion in cellulose (Spiridon *et al.*, 2011). Similarly, the absorption spectrum band at 1639.84 cm^{-1} is assigned to the –OH bending. As shown in Figure 5.4 B, the absorption spectrum band at 600 – 1000 cm^{-1} was strong for untreated BC but somehow is weak for the treated BC material.

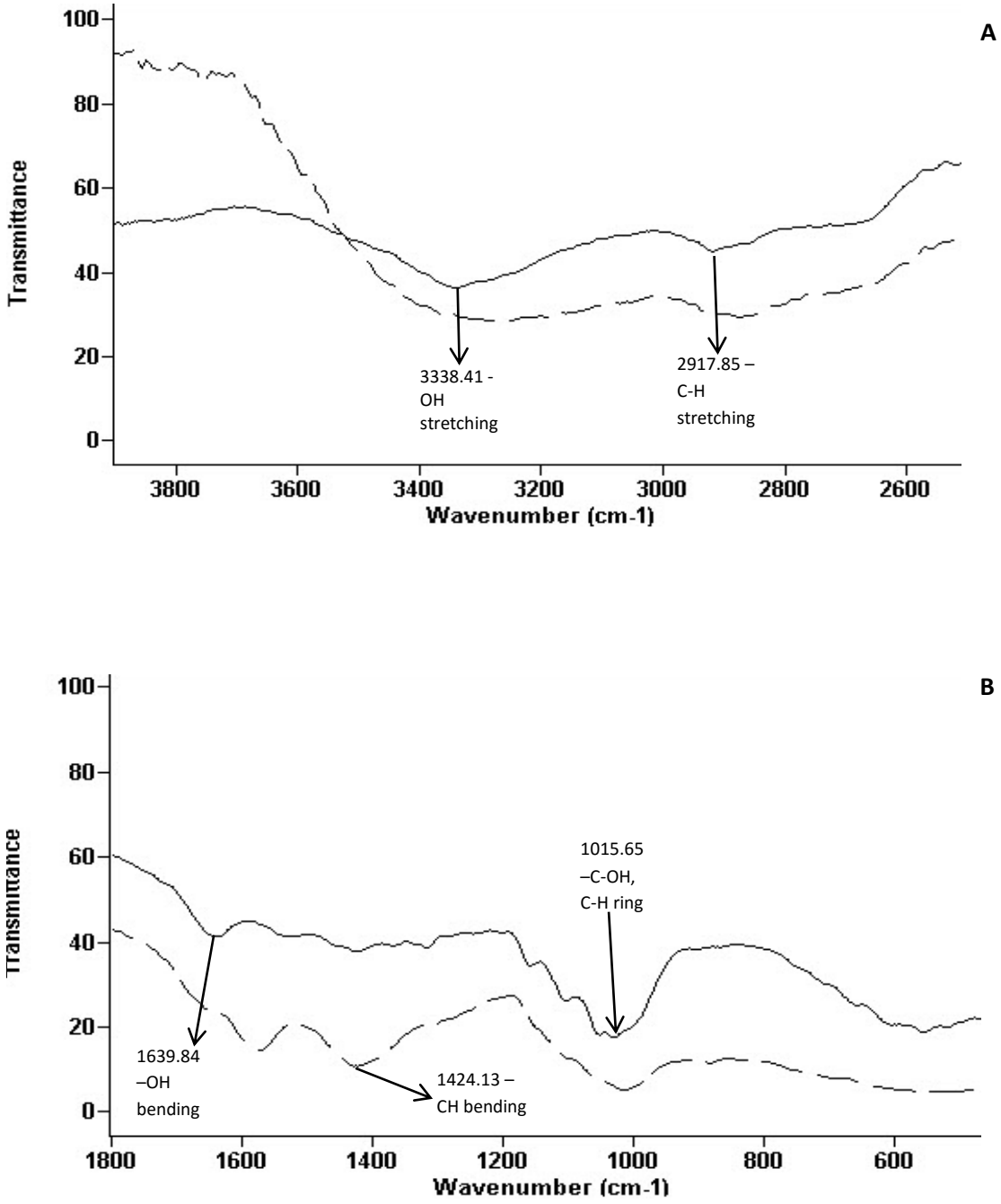


Figure 5.4: FTIR spectrum of BC from *G. xylinus* 639 before and after pretreatment with cellulases. A: 3800 – 2600 cm $^{-1}$ region; B: 1800 – 600 cm $^{-1}$ region. - - - - - Treated BC, ——— Untreated BC.

5.3.2 Scanning Electron Microscopy (SEM) Analysis

SEM analysis was carried out with 4 different samples of BC after freeze-drying. Figure 5.5A shows the SEM micrograph of unwashed BC microfibrils and the bacterial cells enmeshed in it. Figure 5.5B is an SEM image of threadlike cellulosic microfibrils without bacterial cells in them. The fibrils are tightly packed with dendritic nodes present which are thought to be regions of amorphous cellulose. The tightly packed fibrils conferred morphological features similar to pure microcrystalline cellulose. There were also regions of highly crystallized cellulose which contained fibrils that are more defined and orientated. Figure 5.5C shows the SEM image of cellulase-treated BC that was hydrolyzed to sugar monomers. The micrograph shows that some microfibrils are separated while others cluster together. Figure 5.5D shows the SEM image of a non-microbial cellulose sample (Whatman) which differs considerably from the BC; exhibiting a discontinuous and fragmented formation. The non-microbial cellulose shows cellulose sheets having large gaps between the fibers and the fibers are not intertwined closely, while the BC has much smaller fibers which are closely intertwined.

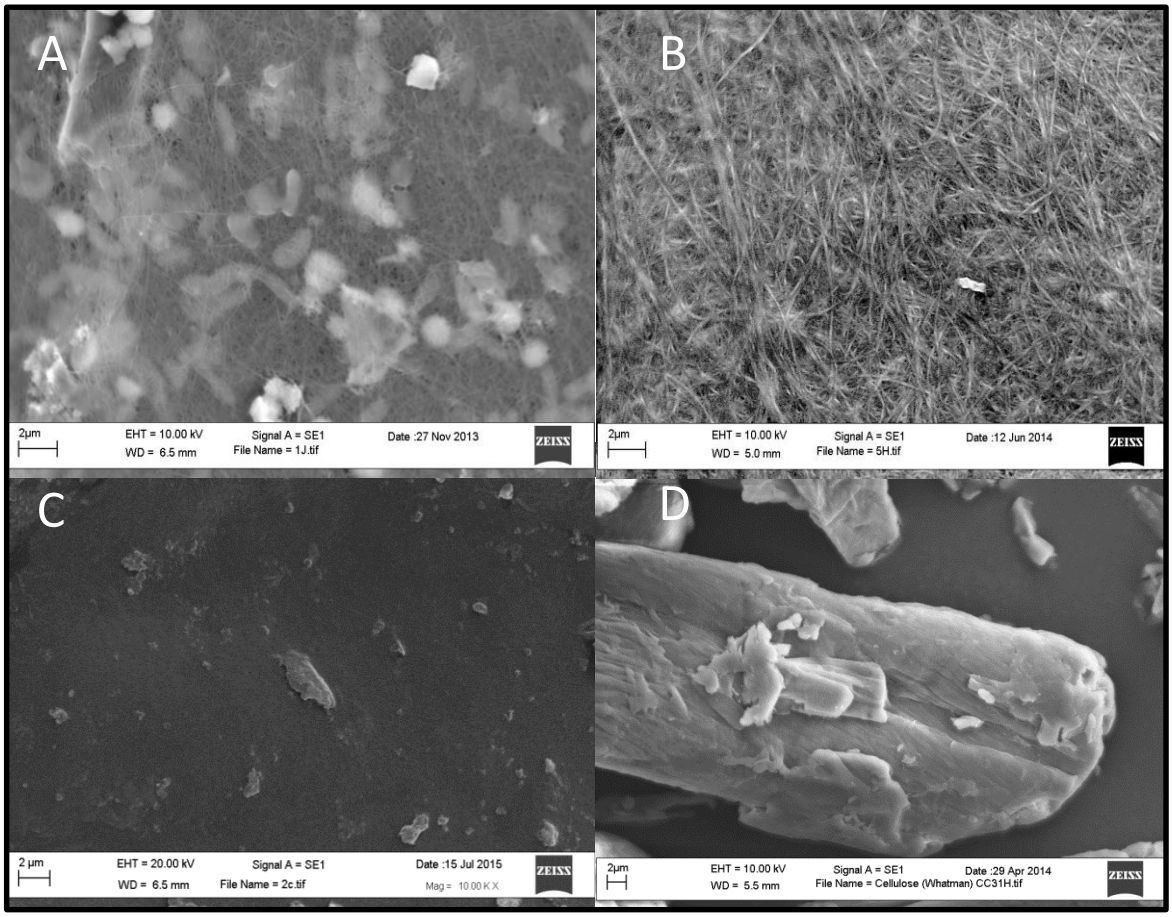


Figure 5.5: SEM collection of BC micrograph from *G. xylinus*. A: Rod-shaped cells of *G. xylinus* (approx 1.5-2µm in length) entangled in BC, B: Cellulosic fibrils of BC, C: Cellulase treated BC (2 mg/ml), D: The non-microbial cellulose sample from Whatman.

5.3.3 X-Ray Diffraction (XRD) analysis

Figure 5.6 shows the XRD pattern of untreated BC. The X- ray diffraction spectra were recorded using an Empyrean PANalytical diffractrometer system in steps of 0.1° using Cu Kα radiation as X-ray source at 25^{o}C. X- ray diffraction spectra were recorded using diffractometer at a plate current intensity of 40 mA and an accelerating value of 40 kV. Scans were performed over the 5.0064 – 79.9904 [^{o}2Th] range using step 0.0130 in width.

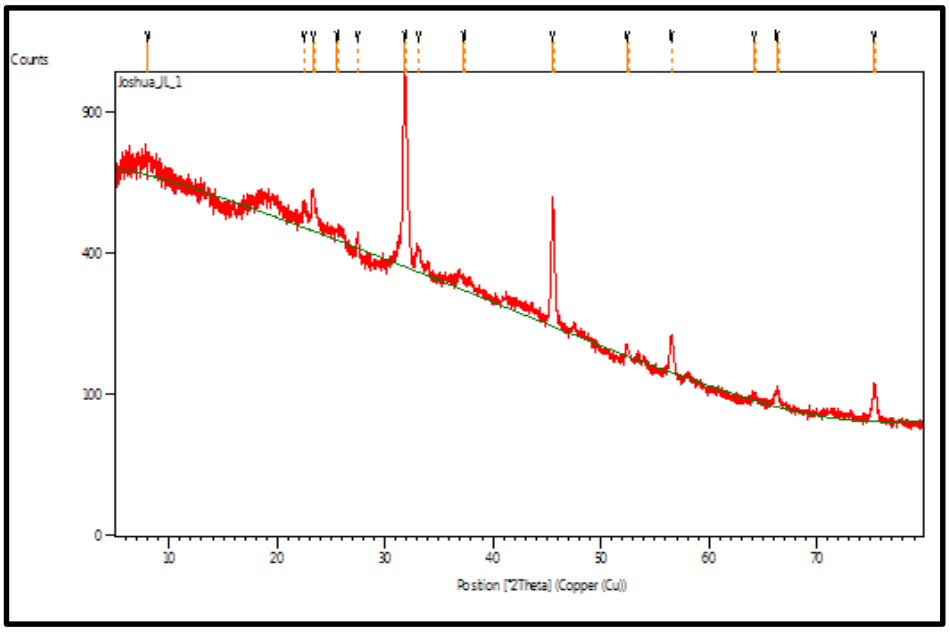


Figure 5.6: XRD diffraction result of dH_2O washed BC from *G. xylinus* 639.

A typical X- ray diffractogram obtained from the BC sample demonstrates two characteristic clearly resolved peaks. The crystallinity index was estimated based on the diffractogram. X-ray diffractogram investigations indicated that the majority of the cellulose was type-1 cellulose (that is crystalline in nature).

5.3.4 Solid State Nuclear Magnetic Resonance (SSNMR) spectroscopy

To determine the content and purity of untreated- and cellulase-treated BC, solid state ^{13}C NMR was carried out using Varian VNMRS 600 spectrometer. Figure 5.7 shows the spectrum from an untreated BC sample. The BC untreated is dominated by a set of signals consistent with cellulose. Judging from the intensity of 89 ppm peak to the broader one at 84 ppm, the BC is quite highly crystalline. Figure 5.8 shows the spectrum of cellulase treated BC sample.

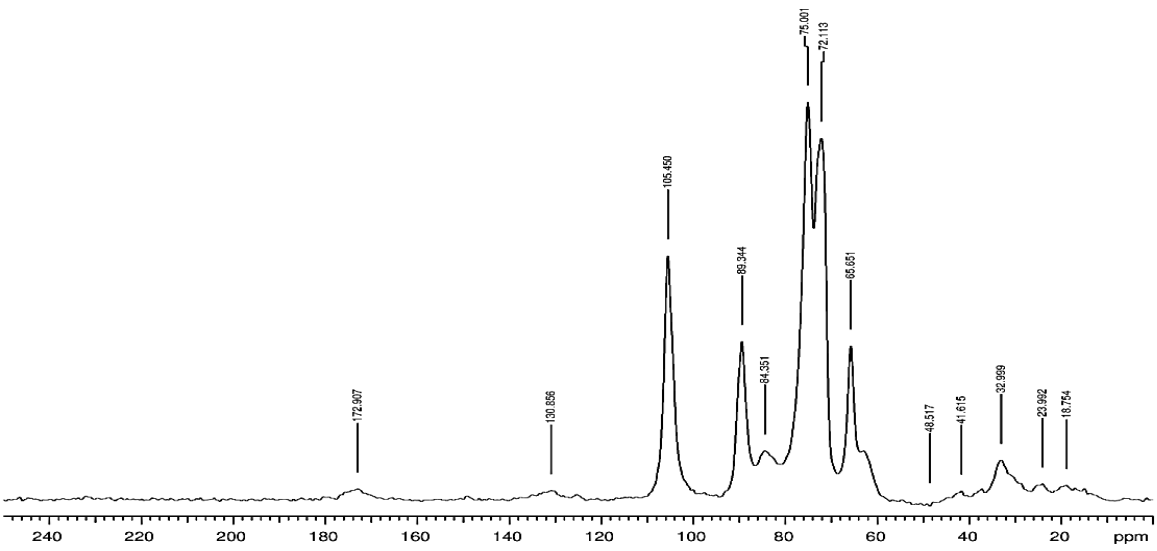


Figure 5.7: ^{13}C SSNMR spectrum of untreated bacterial cellulose (BC) from *G. xylinus* 639

The spectrum is still dominated by the polysaccharide signal between 55 and 110 ppm but it is much less well defined than for the untreated BC sample. The spectrum also shows some other signals at the low-frequency end of the spectrum and four sharp lines at the high frequency end that were not present in the untreated BC sample.

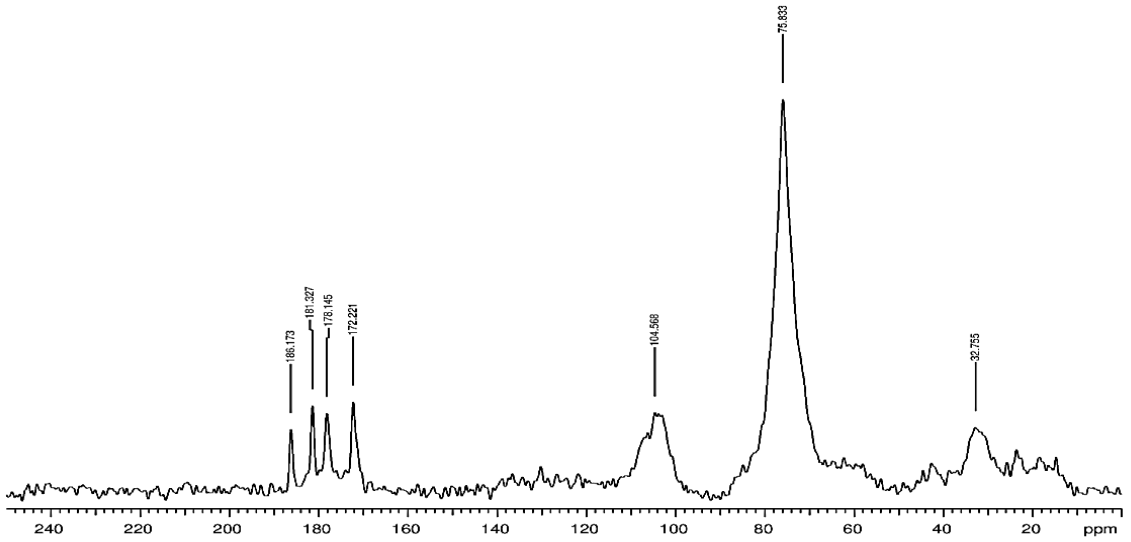


Figure 5.8: ^{13}C SSNMR spectrum of cellulase treated bacterial cellulose (BC) from *G. xylinus* 639

5.3.5 Differential Scanning Calorimeter (DSC)/Thermogravimetric Analysis (TGA)

To determine the thermal transitions of untreated and cellulase treated BC, glass transition (Tg) and melting temperature (Tm) were evaluated. The DSC of the untreated BC indicated that the Tg and Tm were around 38 ^{o}C and 117.31 ^{o}C respectively (Figure 5.9). The BC sample was stable up to 200 ^{o}C above which decomposition started with a pronounced disintegration at 338.13 ^{o}C. The initial BC sample varied greatly between 10 – 200 ^{o}C as a result of evaporation due to water content.

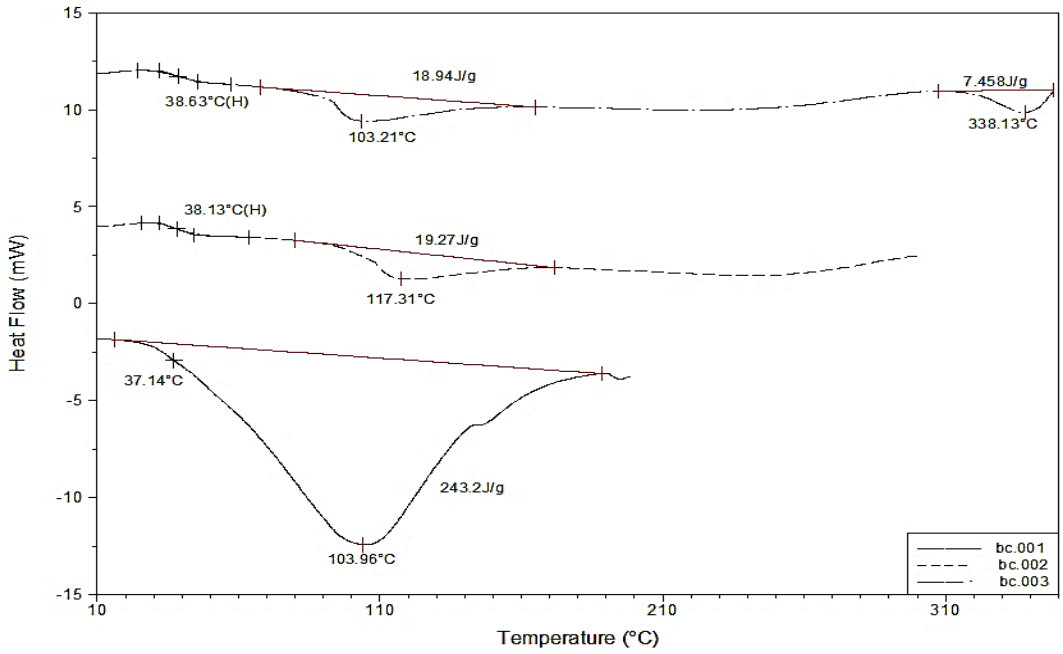


Figure 5.9: DSC thermogram of untreated BC from *G. xylinus* 639 illustrating Tg and Tm

On the other hand, the Tg of cellulase treated BC samples were around 115 ^{o}C (Figure 5.10). Melting transition was not observed. A small endotherm probably due to solvent evaporation was observed at 44 – 60 ^{o}C. There was quick evaporation of the sample which was stable up to around 150 ^{o}C above which disintegration began and there was pronounced decomposition at 170 ^{o}C.

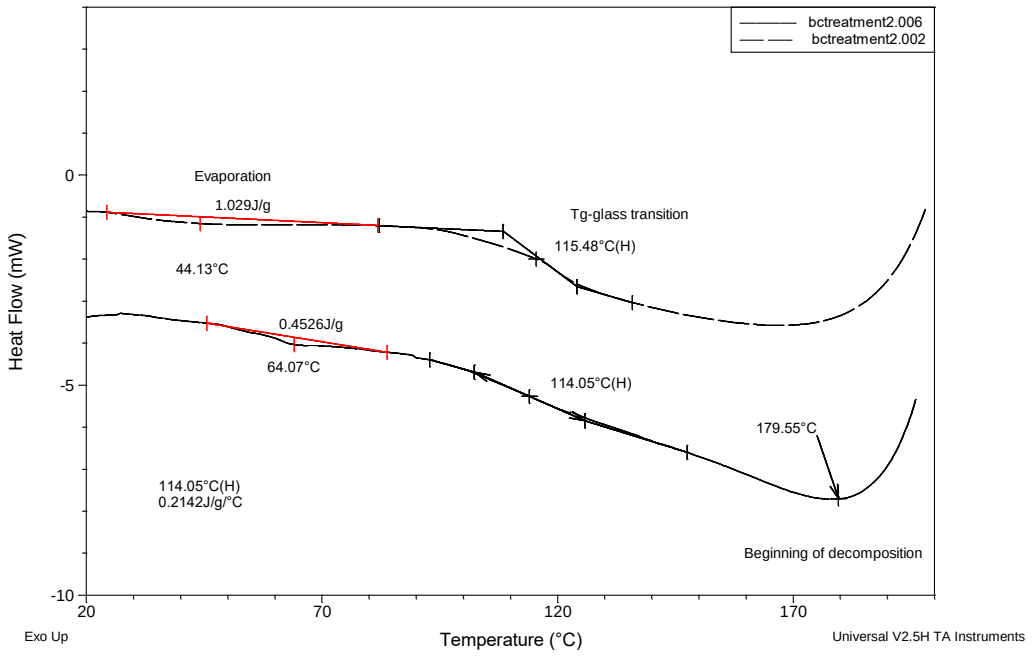


Figure 5.10: DSC thermogram of cellulase treated BC from *G. xylinus* 639 illustrating Tg and Tm

Figure 5.11 shows the TGA curve for the untreated BC sample. The figure shows bound water for the untreated BC sample to be evaporated at approximately 50 ^{o}C with degradation temperature after 200 ^{o}C. Figure 5.12 shows the TGA profile of cellulase treated BC. The figure shows a fast evaporation of the treated sample at a temperature of 30 – 80 ^{o}C which could be ascribed to the presence of moisture and other materials used in the treatment of BC sample. The treated sample was stable until about 150 ^{o}C before degradation. The maximum mass loss for the cellulase treated BC sample was much higher than that of the untreated BC sample.

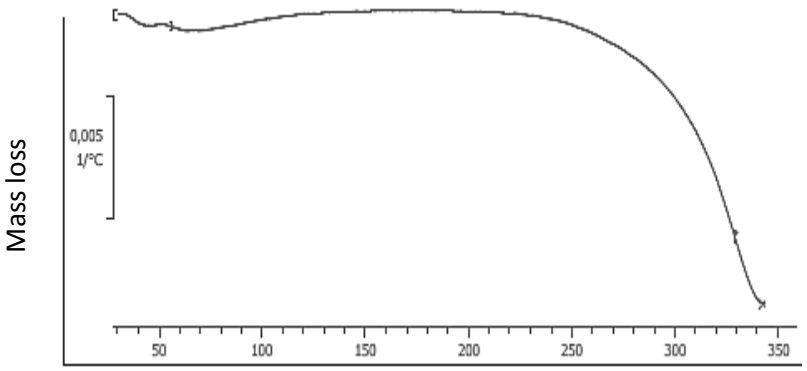


Figure 5.11: TGA thermogram of untreated BC sample from *G. xylinus* 639

Temperature (°C)

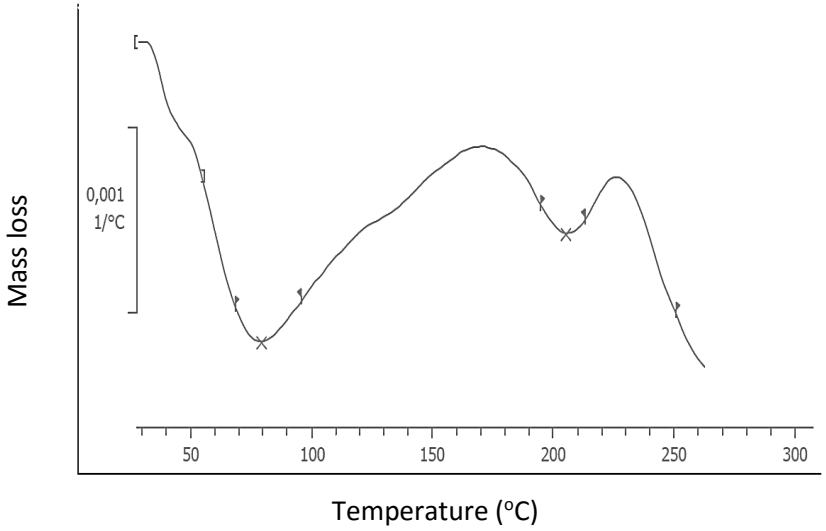


Figure 5.12: TGA thermogram of cellulase treated BC sample from *G. xylinus* 639

5.3.6 GDNA isolation and PCR amplification of *G. xylinus* β-glucosidase genes

The genomic DNA preparation from *G. xylinus* was successful and the PureLink™ Genomic DNA Mini kit yielded high molecular weight genomic DNA which was used as template for PCR reactions. PCR amplification was also successful. The sample was electrophoresed beside an appropriate molecular weight marker. The gel images were digitally captured. Amplicons obtained were sharp and clear indicating good products (Figure 5.13), which were used for cloning after purification of the fragments from the gel.

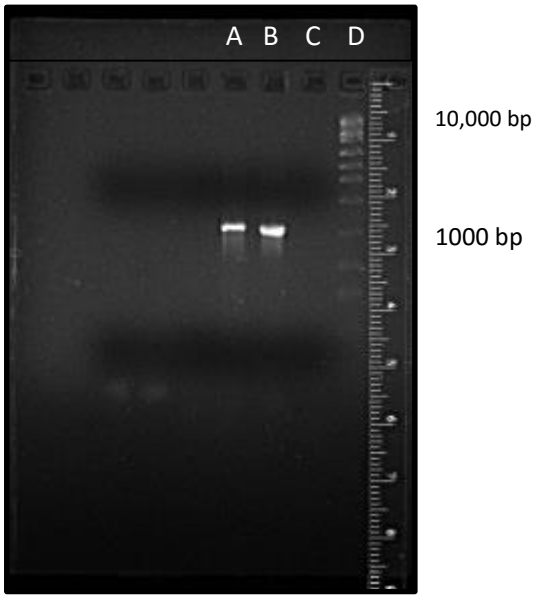


Figure 5.13: Amplification of genomic DNA of *G. xylinus* (AP012159.1) using PCR technique. Lane: A and B – genomic DNA amplicons, C – DNA free control, D - High ranger ladder. PCR products are approximately 1087 bp.

5.3.7 Cloning and sequencing of *G. xylinus* β-glucosidase gene

Approximately 1087 bp PCR product in length was obtained using *G. xylinus* gDNA as a template. PCR amplicons of β-glucosidase genes were gel purified and cloned as described in section 6.2.7. Figure 5.14 shows *E.coli* transformants of pGEM-T clones of *G. xylinus* β-glucosidase PCR fragments selected on ampicillin plates. Approximately 94 clones were obtained (Figure 5.14) and amplification of an appropriate pDNA fragment by PCR was

conducted in one colony to confirm the presence of the right insert. Plasmids were isolated from transformants and were purified using a Qiagen^{R} plasmid plus midi kit. The plasmid DNA was prepared from a cultured cell originating from the colony and it was diluted to 10^{-1}, 10^{-2} and 10^{-3} fold, and used as a template DNA for PCR using same primers used for amplifying genomic DNA.

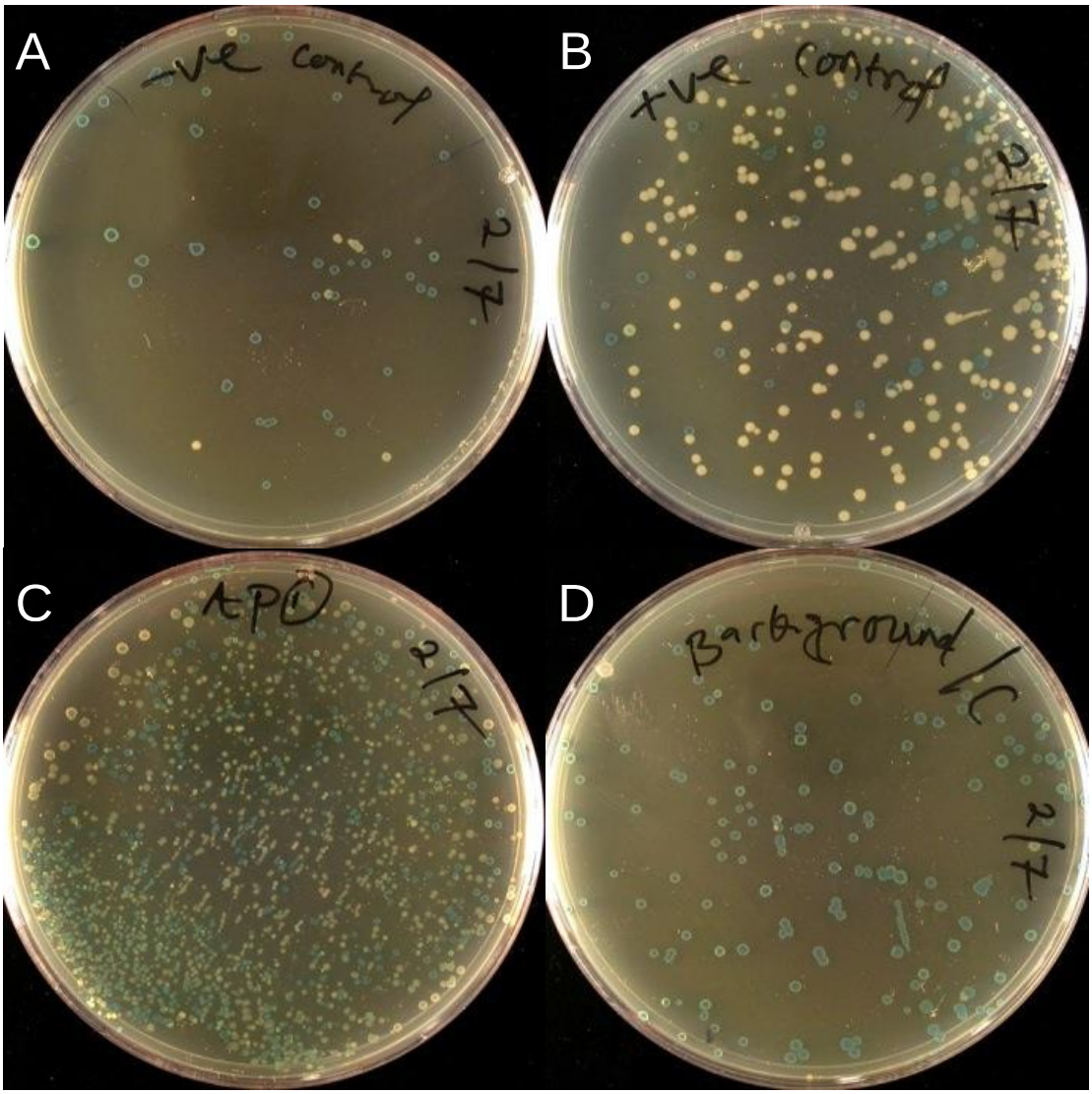


Figure 5.14: *E.coli* transformants of *G. xylinus* β-glucosidase gene selected on ampicillin plates. Potential clones are represented by white colonies where the *lacZ* reporter gene has been disrupted. A: Negative Control (Vector only); B: Positive Control (Vector + control insert + ligase); C: *Gluconacetobacter xylinus* β-glucosidase (AP012159.1) clones; D: Background Control (Vector + ligase).

Figure 5.15 shows the electrophoresis gel images of the colony PCR products. The plasmid contained an insert with approximately 1087 bp. The presence of amplicons with identical size from genomic DNA and plasmid DNA confirmed successful cloning of the same sequence from *G. xylinus*.

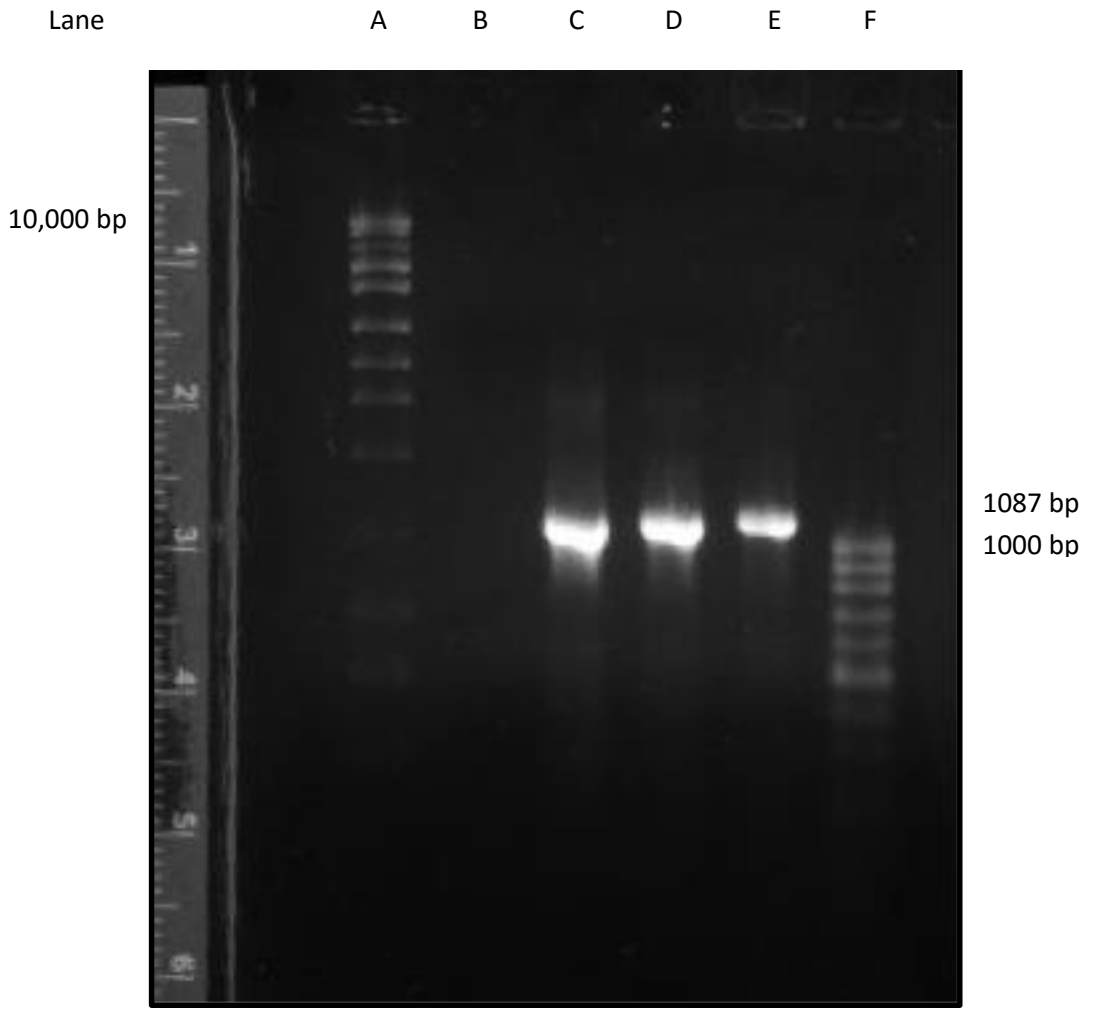


Figure 5.15: Amplification of plasmid DNA of *G. xylinus* β-glucosidase (AP012159.1) using PCR technique. Lane: A - High ranger ladder, B – Control (DNA free); C, D and E – AP012159.1 pDNA (10^{-1}, 10^{-2}, 10^{-3}– pDNA dilutions), F - PCR product ranger ladder. PCR products are approximately 1087 bp.

Figure 5.16 summarises the DNA sequence of the cloned PCR fragment of *G. xylinus* (AP012159.1) pDNA. BLAST analysis confirmed as the nearest significant match (expectation value of 3 e^{-60}) *G. xylinus* 3288 β-glucosidase (AP012159) with 80% identity and 49% coverage.

TAGGTGGTACGACTCCGCGTCCGACTGTGGATATGAATAATCGCAGGACGTGGACGGTGGTATGATGAGG
GGGAGGGATTTCCTTATATTTCTTTAACCGTCATGCTGGATGTGACACCGGTACCCTCTGGTTACAGGATGA
CACATCTGCCTTTGGACGATAACTCACCGAATGGTCCTCCGTTCCGACTGGTTTGCCAGAACCCGTGGTCGA
ACCGGTGCATTGACCCGTAGCGGCTCCCATTATTGGCTAAATTGCTTCGACAACTCCTAATCCCCCGCCTGC
CCCACCCGGACCGCCGCCCCGCTGGGCGGTTGTGGCGCCTGCCCCATCATCACCCTGACCGTGGTGCGATC
CGCCGAACCTGTGGTTGAATCTTCGGGGGGGGTTTCGCGACACCAAAAAAGCGTAGTTCCGCCGTACGTCC
GGTTGTTGCTGCTGTTCTGCGAAGTGCAGGACACCTGAATGGCGCNAACTGAGTTTTTTTGGTGCACGGAA
ACCTGCCTGGAAGATTCAACCACAGGTTCGGTTTCTGCATCCACCACGCTTCGGGCTGATGCGGCAGGCCC
AACCGGCGTATGGCGCCGCGGCGGGTACCGGGAGGGAGATTTGCAAAGAAATTACGATAATGGGGGCCTC
CGGTTGTGATCGGTTCGCCCTGCAACCCGTCGAAGGGAG

Figure 5.16: Nucleotide sequences of *G. xylinus* β-glucosidase (AP012159.1) pDNA

BLAST analysis also indicated that the sequence has 79% identity, $5e^{-53}$ expectation value with 75% coverage to *G. xylinus* D16264.1 β-glucosidase.

5.3.8 Artemis analysis of cellulase gene region

Table 5.3 shows the coding sequence (CDS) sequence analysis of *G. xylinus* NBRC3288 cellulase operon which was performed using Artemis software (https://www.sanger.ac.uk/resources/software/artemis/). Protein gene products identified with Artemis were used to formulate a Blastp search of the non-redundant database at NCBI (www.ncbi.nlm.nih.gov). It is important to note under the coding sequence how some of the genes get across each other by a few bases. Endoglucanase and endoglucanase/β-glucosidase which are both hydrolysing enzymes relate with each other while cellulose synthase catalytic subunit, cyclic di-GMP binding protein (cellulose synthase regulatory), cyclic di-GMP binding protein, cellulose synthase operon protein C and cellulose synthase operon protein D which constitute the cellulose synthase complex also relate with each other by some bases.

Table 5.3: Artemis BLASTp analysis of *G. xylinus* NBRC3288 cellulase complex analysis on NCBI

Gene product	Coding sequence (CDS)	Nearest strain match	Accession No.	Query cover (%)	E value	Identity (%)
Endoglucanase	1 --- 990	*G. medellensis*	WP_014106411.1	100	0.0	100
Endoglucanase/ β-glucosidase	786 --- 2069	*G. xylinus* NBRC3288	BAK84917.1	77	0.0	99
Cellulose synthase	2210 --- 4492	*G. medellensis*	WP_014106413.1	98	0.0	100
Cellulose synthase regulatory subunit	4377 --- 6035	*G. xylinus* NBRC3288	BAK84919.1	92	0.0	100
Cellulose synthase regulatory subunit	6004 --- 6870	*G. xylinus* NBRC3288	BAK84920.1	97	0.0	1000
Cellulose synthase	6741 --- 10880	*G. medellensis*	WP_014106416.1	96	0.0	100
Cellulose synthase	10835 --- 11350	*G. medellensis*	WP_014106417.1	91	$2e^{-109}$	100
β-glucosidase	11542 --- 13779	*G. medellensis*	WP_014106418.1	98	0.0	100

Table 5.4 shows the Blastx of CDS on the complementary sequence of the *G. xylinus* NBRC3288 cellulase operon which was also performed using Artemis software (https://www.sanger.ac.uk/resources/software/artemis/). The complementary sequence results also show how some of the genes connect to each other by a few bases. The result shows how genes for endoglucanase and endoglucanase/β-glucosidase, which are both hydrolysing enzymes, join with each other while the gene for the cellulose synthase catalytic subunits, which constitutes the cellulose synthase complex, also join with each other by some bases.

A further analysis of *G. xylinus* NBRC3288 gene products on the Artemis genome browser (Figure 5.17) revealed a more accurate organization of the contigs, which can be taken as a guide for further analysis of the individual proteins.

Table 5.4: Artemis BLASTx analysis of *G. xylinus* NBRC3288 cellulase complex analysis on NCBI

Gene product	Coding sequence (CDS)	Nearest strain match	Accession No.	Query cover (%)	E value	Identity (%)
Endoglucanase	1 --- 666	*G. medellensis*	WP_014106411.1	100	1e $^{-157}$	100
Endoglucanase	667 --- 990	*G. medellensis*	WP_014106411.1	100	5e $^{-71}$	100
Cellulose synthase regulatory unit	5935 --- 6354	*G. xylinus* NBRC 3288	BAK84920.1	78	7e $^{-69}$	100
Cellulose synthase	6784 --- 7353	*G. medellensis*	WP_014106416.1	84	2e $^{-97}$	100
β-glucosidase	11521 --- 12456	*G. medellensis*	WP_014106418.1	93	0	100
β-glucosidase	12457 --- 12861	*G. medellensis*	WP_014106418.1	100	3e $^{-83}$	100
β-glucosidase	12919 --- 13779	*G. medellensis*	WP_014106418.1	99	0	100
Endoglucanase/β-glucosidase cellulose synthase subunit	1245 --- 1625	*G. medellensis*	WP_041247425.1	100	4e $^{-86}$	100
Cellulose synthase 1 catalytic subunit	1629 --- 2033	*G.* sp. SXCC-1	EGG76192.1	38	8e $^{-15}$	87
Endoglucanase	2034 --- 2387	*G. medellinensis*	BAK84917.1	100	4e $^{-75}$	100
Cellulose synthase	4473 --- 4967	*G. xylinum* NBRC 3288	BAK84919.1	95	2e $^{-97}$	100
Cellulose synthase	5058 --- 5711	*G. xylinus* NBRC 3288	BAK84919.1	100	8e $^{-150}$	100
Cellulose synthase	5712 --- 6020	*G. xylinus* NBRC 3288	BAK84919.1	100	6e $^{-67}$	100
Cellulose synthase regulatory subunit	6195 --- 6626	*G. xylinus* NBRC 3288	BAK84920.1	99	3e $^{-95}$	100
Cellulose synthase	6687 --- 7298	*G. medellensis*	WP_014106416.1	69	7e $^{-85}$	100
Cellulose synthase	7299 --- 7814	*G. medellensis*	WP_014106416.1	100	1e $^{-109}$	100
Cellulose synthase	8583 --- 9161	*G. medellensis*	WP_014106416.1	100	1e $^{-121}$	100
Cellulose synthase	9450 --- 10610	*G. medellensis*	WP_014106416.1	100	0	100
β-glucosidase	11808 --- 12167	*G. medellensis*	WP_014106418.1	99	6e $^{-73}$	100
β-glucosidase	12486 --- 12851	*G. medellensis*	WP_014106418.1	99	1e $^{-72}$	100
β-glucosidase	12852 --- 13325	*G. medellensis*	WP_014106418.1	99	8e $^{-101}$	100
Endoglucanase	725 --- 1171	*G. medellensis*	WP_014106411.1	78	8e $^{-76}$	100
Endoglucanase	1826 --- 2137	*G. medellensis*	WP_014106411.1	76	9e $^{-46}$	100
Cellulose synthase	2423 --- 3289	*G. medellensis*	WP_014106413.1	100	0	100
Cellulose synthase	3290 --- 3613	*G. medellensis*	WP_014106413.1	100	5e $^{-65}$	100
Cellulose synthase	4034 --- 4753	*G. medellensis*	WP_014106413.1	63	1e $^{-98}$	100
Cellulose synthase regulatory subunit	5123 --- 5674	*G. xylinus* NBRC 3288	BAK84919.1	99	3e $^{-121}$	100
Cellulose synthase	6716 --- 7084	*G. medellensis*	WP_014106416.1	56	9e $^{-36}$	100
Cellulose synthase	10859 --- 11743	*G. medellensis*	WP_014106417.1	52	3e $^{-107}$	100

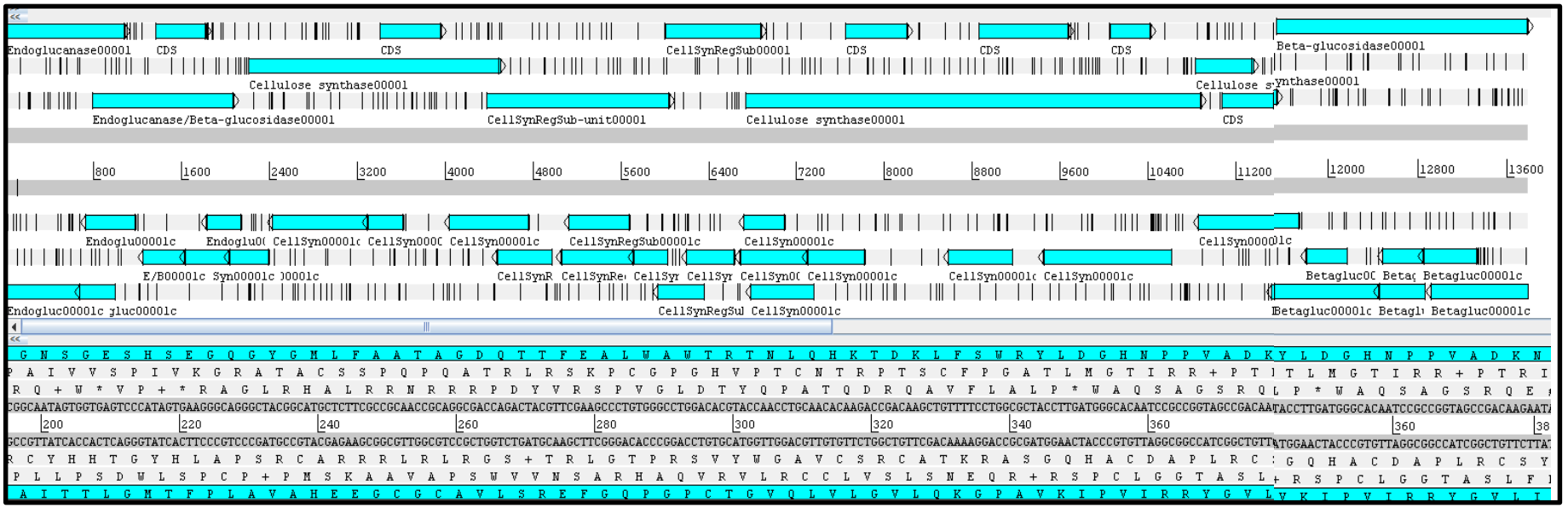


Figure 5.17: Artemis screenshot showing sequence annotation from *G. xylinus* NBRC3288 cellulase complex. The Figure shows a DNA sequence translated into its six reading frames with vertical bars indicating stop codons. The view also shows an overview of the cellulase synthase gene.

5.4 Discussion

5.4.1 Production of bacterial cellulose

After nine days of static fermentation, bacterial cellulose (BC) was obtained as a thick layer on the surface of HS medium. The yield of BC produced by *G. xylinus* was found to be 4.2 g/L weight with an average dry mass of 0.21 g. The thickness of the wet BC was 12 mm and the thickness of the dry sheets was 0.18 mm. The yield results of BC production in this study fall within the previous work of Carreira *et al*., (2011) and Gayathry and Gopalaswamy (2014) who reported BC production with a yield of 2.60 g/L and 11 g/L respectively. Differences in yield may reflect strain differences.

5.4.2 Fourier Transform infrared spectroscopy FTIR

Figure 5.3 shows the FTIR absorption spectrum of standard cellulose and BC. The FTIR results as deduced from Table 5.1 indicated many similarities between the standard and bacterial cellulose. Results indicated that all samples contain functional groups that were associated with cellulose, such as: 3700 - 3000 cm^{-1} hydrogen bonding -OH bending, 2970 - 2800 cm^{-1} -CH symmetrical stretching and 1034 - 1023 cm^{-1} C-C, C-OH and C-H ring side group vibrations, which is typically widely reported for bacterial cellulose (Trovatti *et al*., 2011; Fan *et al*., 2012).

Generally, the adsorption of enzymes on to the surface of their substrates is assumed to be an essential step for hydrolysis (Bernd *et al*., 1994). The broad absorption spectra in the region 3600 - 3000 cm^{-1} (-OH stretching, H-bonded) indicates a shallower peak for the treated BC sample. Treatment with cellulase enzyme is thought to disrupt the β (1→4) linkages between the cellulose chains (Fan *et al*., 2012). Hydrolysis of the β-linkages can lead to changes in the molecular, supramolecular and morphological level orientation (Fan *et al*., 2012) which can have a variety of effects on the physical properties of the BC such as crystallinity, water holding capacity and mechanical strengths. The spectra in Figure 5.3 and 5.4 (A and B) indicate that the untreated BC consists of type I cellulose. Cellulose I

and II are found in nature with cellulose I as the main form found in nature and it occurs as two allomorphs denominated Iα and Iβ (Festucci-Buselli *et al*., 2007). Type II cellulose is the most crystalline thermodynamic stable, and can be obtained from cellulose I by regeneration and mercerization (Festucci-Buselli *et al*., 2007).

The -OH bending of adsorbed water was observed at 1639.84 cm^{-1} for untreated BC samples. This result is in agreement with the report of Okiyama *et al*., (1992) whereby 0.3% of the 90% of water (by mass) is held through the extensive hydrogen bonding network facilitated by the hydroxyl groups and ester bonds. In untreated BC samples, the intensity for adsorbed water is comparably less intense than those found in cellulase treated BC sample spectra. The FTIR absorption spectra for both treated and untreated BC show characteristic peaks around 1000 – 1200 cm^{-1} (Zhbankov *et al*., 2000; Langkilde and Svantesson, 1995) and the band around 1424.13 cm^{-1} in treated BC can be ascribed to CH shake vibrations in cellulose (Spiridon *et al*., 2011). Generally, the results of the FTIR investigation in Figure 5.4 A and B showed that the peaks of BC samples were lower after enzymatic hydrolysis indicating that the BC was degraded. The band at 1400 – 1600 cm^{-1} for the treated BC sample could be attributed to the absorbed water bending vibrations after enzymatic hydrolysis.

5.4.3 Scanning Electron Microscopy (SEM) Analysis

Confirmation of the general structure of the untreated and cellulase treated BC was carried out using the SEM analysis. BC images from SEM micrographs (Figure 5.5) revealed complex meshed cellulose ribbons that interweaved among each other to form the BC membrane. Figure 5.5A is an SEM image of BC washed with dH_2O showing distinct rod-shaped cells that were immobilized and entangled by the fine cellulose ribbons. The length of each cell was roughly 1 – 2 µm. The regions containing thicker cellulose ribbons displayed no obvious sign of cells, which suggests that the cells were covered by the successive formation of cellulose and that these fine fibrils were in the early stages of

cellulose matrix formation. The absence of other cell types indicates that *G. xylinus* is the only producer of the BC pellicle. Interestingly, samples that were subjected to repeated and prolonged washing with dH_2O showed no presence of cells within their matrix thus suggesting that thorough washing of BC with dH_2O is effective in removing cells and other cellular debris.

Figure 5.5B shows the SEM image of threadlike cellulosic microfibrils of BC. White dendritic nodules were observed throughout the structure of the BC, forming junctions, diverging microfibers from the center which were then incorporated into the surrounding meshwork. The dendritic nodes which were widespread throughout the sample in a variety of shape and sizes were amorphous in nature indicating the presence of type II cellulose (Sarkar and Perez, 2012). Whilst washing of BC with dH_2O was effective in removing cells, treatment of the sample with NaOH before washing with dH_2O may be successful in the context of sterility. A dramatic morphology change on BC microfibrils was observed after hydrolysis (Figure 5.5A) indicating that BC microfibrils were hydrolyzed to sugar monomers.

5.4.4 X-Ray Diffraction (XRD) Analysis

Figure 5.6 showed the XRD curves of dH_2O washed BC. The XRD analysis result gave an insight into the physical properties of the BC produced. Bacterial cellulose diffractogram reveals two principal diffraction peaks at 15^o and 29^o confirming the presence of type-1 cellulose (Czaja *et al*., 2004; Moosavi-Nasab and Yousefi, 2011; Sheykhnazari *et al*., 2011). Their studies indicated that a high intensity at diffraction plane 14.5^o and 22.6^o indicates the presence of cellulose type-1.

5.4.5 Solid State Nuclear Magnetic Resonance (SSNMR) spectroscopy

The ^{13}C SSNMR spectrum of untreated BC is presented in Figure 5.7. The broader peak was due to the crystallite surfaces and the amorphous domains while the comparatively sharp peak was due to a crystalline region (Shezad *et al*., 2010). The NMR spectrum of the

BC is in agreement with those reported in the literature for BC and cellulose (Shezad *et al*., 2010; Zhao *et al*., 2007). Although the SEM analysis (Figure 5.5 C) shows dramatic changes on cellulose microfibrils of cellulase treated BC sample, the NMR data (Figure 5.8) were still dominated by polysaccharide signals. The NMR result of the cellulase treated BC (Figure 5.8) shows that BC apparent crystallinity was alternated by hydrolysis which suggests that BC could be a valuable raw material for fermentable sugars.

5.4.6 Differential Scanning Calorimeter (DSC)/Thermogravimetric Analysis (TGA)

The DSC thermogram of thermal behaviour including Tg and Tm of untreated BC and cellulase treated BC are shown in Figure 5.9 and 5.10 respectively. The thermal behaviour evaluated by DSC and TGA shows the thermal stability of BC. DSC measures the heat absorbed or released by a material as a function of temperature or time. The Tg of the untreated BC was found to be around 37.14 ^{o}C which was found to be lower than the value 44.28 ^{o}C reported by Mohite *et al*., (2014) but higher than the value of 13.94 ^{o}C reported by George *et al*., (2005). The higher the Tg of a material, the better the advantage because minimal aging is expected at storage temperature which is below Tg (Bechard *et al*., 1995). The thermal stability of BC up to 200 ^{o}C could be attributed to its crystallinity and high molecular weight (Chen *et al*., 2009) while the low thermal stability of treated BC could be as a result of enzyme hydrolysis yielding low molecular weight oligosaccharides. The results indicated that the cellulase treated BC sample was hydrolysed; hence the low Tm and decomposition temperatures compared to the untreated BC samples.

Thermal degradation temperature of a sample is influenced by the following factors: sample size, moisture content of sample and the stability of functional groups. Other features include experimental factors such as nitrogen flow rate and the heating rate (Chang *et al*., 2010; Roman and Winter, 2004). The TGA results (Figure 5.11 and 5.12) confirm the evaporation in both untreated and treated BC samples which is caused as a result of solvent or the presence of water in the BC samples. The probable presence of

water could be as a result of sample exposure which thereby absorbs atmospheric moisture. The results from the TGA analysis also confirm the degradation of untreated BC after 200 ^{o}C and that of treated BC after 150 ^{o}C. The fast degradation of cellulase treated BC in DSC analysis was found to be consistent with the observed degradation in TGA analysis.

5.4.7 Cloning, Sequencing of *G. xylinus* (AP012159.1) β-glucosidase insert and BLAST results

Literature searches show that a number of β-glucosidase genes have been cloned from *G. xylinus* (e.g. Tajima *et al*., 2001; Kawano *et al*., 2002). Manual analysis of the nucleotide sequence open reading frame indicated the sequence to be truncated by several stop codons (data not shown), suggesting that the translated protein may be non-functional. The reasons why *G. xylinus* produces β-glucosidase, which is an enzyme that catalyses the hydrolysis of cellobiose to produce glucose are not very clear. Tajima *et al*., (2001) reported that the addition of a large amount of β-glucosidase (100 μg/ml) from sweet almond enhances BC production. It is also documented that in addition to hydrolysis of oligosaccharides, β-glucosidase is also involved in transglycosylation (Kono *et al*., 1999). These observations suggest the possibility that *G. xylinus* β-glucosidase plays a role in regulating glucose concentration and also oligosaccharides, which are the materials for cellulose production, whilst also regulating the expression of other genes and proteins (Kawano *et al*., 2002). It may recycle cellobiose molecules back into glucose for reincorporation into new cellulose molecules.

5.4.8 Artemis analysis of the cellulase gene region

In this study, Artemis was used to visualize the cellulose synthase gene region from *G. xylinus* NBRC 3288. The cellulose synthase complexes are located on the longitudinal axis of the cytoplasmic membrane and are coded by the cellulose synthase operon which code for different subunits (Wong *et al*., 1990). The cellulose synthase operon system and machinery is described in section 5.1 and Figure 5.17 shows cellulose synthase to be

flanked by endoglucanase and β-glucosidase hydrolytic proteins suggesting that these enzymes play a role in *G. xylinus* cellulose synthesis. The proteins involved in the cellulose biosynthetic process exist as a complex mediating the various steps of BC synthesis (Whitney *et al*., 2011). The operon structure of cellulose synthase has been elucidated by Wong *et al*., (1990) by genetic complementation of *G. xylinus* 1306. The operon has been further characterized by Saxena *et al*., (1994) to elucidate the function of the protein encoded by each gene of the operon using site-directed insertional mutagenesis of *G. xylinus* ATCC 53582 strains. Deng *et al*., (2013) found that the disruption of *acsA* affected the production of AcsB and AcsC but not the production of Acsd, while the disruption of *acsC* resulted in the production of truncated AcsC but did not affect the production of AcsAB or AcsD.

5.5 Conclusion

BC obtained from *G. xylinus* could be of commercial interest and open new avenues in the field of degradable polymers (Andrade *et al*, 2010). In this study, BC was produced using Hestrin-Schramm (HS) media. FTIR investigation indicated the presence of CH_2 and OH group at the absorption wavelength of 3300 cm^{-1}, 1650 cm^{-1} and 1025 cm^{-1} indicating that the properties of BC are close to that of pure cellulose. FTIR investigation evidenced that the peaks of BC were lower after enzymatic hydrolysis. SEM results showed fine morphology of the cellulose matrix and fibre; and XRD indicated the majority of the cellulose to be type-1 cellulose. NMR result of the untreated BC confirmed the crystallinity of BC. Though morphology changes on BC were observed after hydrolysis with cellulase enzyme, no significant changes were detected in the FTIR patterns. In the TGA study, the influence of sample moisture content was present only at temperatures below 100 ^{o}C and the weight loss of the hydrolysed BC was faster than the untreated sample. In conclusion, this study provides for the first time a fully detailed characterisation of pure bacterial

cellulose before and after treatment with a commercially available cellulase enzyme. A novel β-glucosidase from *G. xylinus* was also successfully cloned and sequenced. This suggests an attractive prospect for the production and recycling of biopolymers from bacterial sources.

Chapter 6

Cloning of hydrophobin genes, cloning and expression of β-glucosidases

6.1 Introduction

The degradation of cellulolytic materials into simple sugars is usually carried out by the treatment of lignocellulosic materials with hydrolytic enzymes. However, other non-catalytic proteins such as hydrophobin may also play a significant role in the disruption of the non-hydrolytic crystalline substrate (Valdeir and Jack, 2010). The purification of enzymes or proteins is necessary for detailed studies on their properties. In Chapter 1(section 1.1 and section 1.2.8), the properties of β-glucosidases and other hydrolytic enzymes is discussed in details. Chapter 1 (section 1.2.6) also describes the properties of hydrophobins and it potentials in cellulose degradation.

There are so many instances in the literature on the cloning of β-glucosidases from bacterial and fungal sources (Nair *et al*., 2013; Karnaouri *et al*., 2013; Li *et al*., 2014). A β-glucosidase-like enzyme encoding gene (*bglH*) of *Bacillus pumilus* (CL16) was cloned by Bogas *et al*., (2007) in *Escherichia coli*. The complete nucleotide sequence open reading frame of 1419 bp had 472 amino acid residues without a characteristic signal peptide sequence suggesting that the enzyme is intracellular. Karnaouri *et al*., (2013) cloned a β-glucosidase gene (*bgl3a*) from *Myceliophthora thermophila* and were expressed in *Pichia pastoris*. The recombinant β-glucosidase with a molecular weight of 90 kDa was purified and characterized. The optimal pH and temperature of the enzyme on pNPG was 5.0 and 70 ^{o}C respectively, with a Km of 0.39 mM on pNPG and 2.64 mM on cellobiose. The use of *Pichia pastoris* system in the expression of recombinant β-glucosidases from *Chaetomium thermophilum* CT2 is reported by Xu *et al*., (2011). The full-length cDNA of the β-glucosidase contained an open reading frame of 2604 bp nucleotides and encoded 867 amino acid residues with a potential secretion signal peptide.

In a recent study, Uchiyama *et al*., (2015) applied a metagenomics approach to screen glucose-tolerant β-glucosidase retrieved from a Kusaya gravy (a kind of brine used for the production of special salt-dried fish in Japan) metagenome. They created a metagenomics

library in *Escherichia coli* and seven glucose-tolerant clones were identified, each of which contained a single *bgl* gene. The deduced amino acid sequences of these genes gave 452 amino acid residues and were found to belong to the Glycosyl hydrolase family 1.

Ng *et al*., (2000) isolated two genes encoding hydrophobins (Le.hyd1 and Le.hyd2) from a primordial cDNA library of *Lentinula edodes* (an edible mushroom native to East Asia) and the nucleotide sequences were determined. These two genes were 760 and 738 bp in length and the deduced amino sequences were homologous to various fungal hydrophobins with characteristic cysteine spacing. Hydrophobin cDNA (fv-hyd1) which is specifically expressed during fruiting body development has been isolated from the basidiomycete *Flammulina velutipes* (Masato *et al*., 2005). Analysis of the genome structure of fv-hyd1 revealed an open reading frame composed of 363 nucleotides and interrupted by three introns. The deduced amino acid sequence showed similarity to those of other fungal class I hydrophobins and contained eight cysteine residues highly conserved among hydrophobin proteins. Leger *et al*., (1992) also determined the sequence of a starvation-stress gene (*ssgA*) of the entomopathogenic fungus *Metarhizium anisopliae* and deduced it amino acid. The primary structure of the SSGA (96 amino acid) protein shares extensive similarities with fungal wall proteins of the hydrophobin and the eight cysteine residues and the putative signal sequences were conserved. Details on hydrophobins and their heterologous expression using *Pichia pastoris* are discussed in Chapter 1 (section 1.2.6) and Chapter 4 (section 4.1).

To attain a systematic lignocellulose degradation process, a good grasp and understanding of the relevance, properties and specificity of these catalytic and non-catalytic proteins is needed (Perez-Fuentes *et al*., 2014). Generally, the method of recombinant protein expression involves the construction of plasmid that encodes the desired protein, insertion of foreign gene into an expression vector, followed by the introduction of the vector into

the required host cells such as the genome of *P. pastoris* and finally inducing protein expression (Cereghino and Cregg, 2000).

The aim of this chapter is to:

i. Clone and sequence novel hydrophobins from *A. nidulans*.

ii. Clone and express β-glucosidases from *Aspergillus* constructed by direct gene synthesis.

iii. Clone β-glucosidases from *Gluconacetobacter xylinus*.

6.2 Materials and methods

6.2.1 Molecular Biology kits

PCR product purification kits, QIA quick Gel Extraction kits and Plasmid DNA extraction kits were all supplied by QIAGEN. PCR Master Mix, pGEM^{R}-T Easy vector system II and JM109 Competent cells (> 10^{8} cfu/µg) were obtained from Promega. EasySelect^{TM} *Pichia* Expression kits, PureLink^{R} Genomic DNA Mini kits and Yeast nitrogen base (YNB) were products of Invitrogen. Afu6g12010 and NFIA_027390 synthetic genes were constructed by GeneArt/Life technologies (Invitrogen, 2013, 2014; lifetechnologies.com/genesynthesis). Midori Green Advance stain, High Ranger 1 kb DNA ladder and PCR products ladder were obtained from Geneflow, UK.

6.2.2 Synthetic plasmid DNA description

The synthetic β-glucosidase gene NFIA_027390 (Chapter 3, section 3.3.5) was assembled from oligonucleotides and the fragment was cloned into pMK-RQ (kanR) (Appendix 35). The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100% (Invitrogen, 2014).

The synthetic β-glucosidase gene Afu6g12010 (Chapter 3, section 3.3.10) was assembled from oligonucleotides and the fragment was also cloned into pMA (ampR) (Appendix 36). The plasmid DNA was also purified from transformed bacteria and its concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100% (Invitrogen, 2013).

6.2.3 Genomic (g) DNA Extraction

The *A. nidulans* G0281 strain was used in this study and *Escherichia coli* (*E. coli*) JM109 competent cells were used for cloning of the hydrophobin gene from *A. nidulans* G0281. Where kits were used, the manufacturer's instructions were followed.

A. nidulans G0281 genomic DNA was extracted by three different methods:

The Oakley lab method is a slightly modified method of M. Peñava Lab method: spores were collected from the surface of colonies using a wire loop wetted with breaking buffer (2% Triton X-100, 1% SDS, 100 mm NaCl, 1 mm EDTA, and 10 mm Tris-HCl, pH 8) and suspended in 0.1ml of sterile breaking buffer in 1.5ml sterile micro-centrifuge tubes. 150 mg of 0.45 – 0.5 mm sterile glass beads were added and vortexed for 30 seconds. The preparation was incubated at 65^{o}C in a water bath for 30 minutes while vortexing for 30 seconds at 10 minutes intervals. 0.1ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the preparation mixed by vortexing, then centrifuged at 13,500rpm for 5 minutes. A volume of 80µL from the separated upper aqueous phase was collected and transferred into a new micro-centrifuge tube (Hervas-Aguilar *et al*., 2007).

In the second method, spores were suspended in 100µL of breaking buffer contained in a 1.5ml micro-centrifuge tube using a wire loop. The preparation was heated at 95^{o}C in a water bath for 15 minutes, after which it was kept on ice for 1 minute, vortexed for 10 seconds and spun briefly (Liu *et al*., 2011).

The third method involved the use of the freezing method of Xu and Hamer (1995), which was carried out by suspending *A. nidulans* G0281 spores into 5µL of sterile water in a 0.5ml micro-centrifuge tube and freezing the preparation in a -80^{o}C freezer for 10 minutes.

One-half of DNA preparations from each method were precipitated using isopropanol in order to obtain a more purified genomic DNA. This was done by adding 0.54 x volume of DNA preparation of isopropanol and precipitating for 30 minutes in -20^{o}C freezer. The mixture was centrifuged for 10 minutes at full speed (13,000 rpm), the supernatant was

discarded and the pellet was completely air dried on a laboratory tissue. 500µL of 70% ethanol was used to wash the pellet by brief vortexing and re-centrifugation for 2 minutes at full speed. After drying, the pellet was re-dissolved in 5µL TE buffer (10mM Tris base, 1mM EDTA and pH 7.5) and stored at -20^{o}C.

G. xylinus genomic DNA was extracted using PureLink^{TM} Genomic DNA Mini kit (Catalog No. K1820-00) according to manufacturer's instructions. The extracted genomic DNA was used as a template for PCR reactions.

6.2.4 Primer design

PCR was used to amplify and sub-clone target genes from genomic DNA or plasmid constructs. Hydrophobin encoding genes, ANID_05290.1 and ANID_07327 of *A. nidulans* G0281 were selected using DNA sequence data of the Broad Institute's, *Aspergillus* Comparative Database (http://www.broadinstitute.org/). Primers to be used for polymerase chain reactions (PCR) were designed by using the NCBI primer design tool (Appendix 3). An extra 300 nucleotides were added both upstream and downstream of each gene sequence in order to increase the chance of getting sets of primers flanking the full structural gene and regulatory elements.

Primer BLAST from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast) ensures specificity of primers by performing a BLASTn search using the input source DNA. *Aspergillus nidulans* was entered in the 'Organism' field to utilize this feature and the database field was set to nr (non-redundant). The minimum PCR product sizes for ANID_05290.1 and ANID_07327 were set to 600 and 680 respectively considering their individual lengths. The best set of primers was selected based on their product lengths, start and stop positions, melting temperatures (Tm) and GC content (Table 6.1). The best primers were ordered from Sigma (http://www.sigmaaldrich.com).

Specific primers with *EcoR*I restriction sites (Table 6.2) were also designed in order to lift the gene (insert) of interest from plasmid DNA for ligation with the expression vector.

Table 6.1: List of primers designed for PCR amplification of hydrophobins and β-glucosidase genomic DNA from *A. nidulans* (*) and *A. niger* (^) respectively (Sigma-Aldrich)

Serial No.	Sequence accession	Sequence (5' – 3')	Tm (ºC)
1	*ANID_05290.1 - F	CGCACTGATCCACTCGTTCT	60
2	*ANID_05290.1 - R	TAGCAAACACGCAGTAGCCA	60
3	*ANID_07327 - F	GTATGTGGGTGCAACATGGC	60
4	*ANID_07327 - R	CCACCCACAAATCCTGACCA	60
5	^ANRA12.6 -F	GGATTGATCTGGACCCCGTC	59.89
6	^ANRA12.6 - R	ACACATAGCTCAACACCTGC	58.19
7	^ANRA12.9 - F	AGCGGCGTGGTCAATCAATA	60.11
8	^ANRA12.9 - R	AGCGCTCTTGTCACAAACACA	61.01

F: Forward primer; R: Reverse primer

Table 6.2: List of specific primers flanked with *EcoR*I restriction sites designed for PCR amplification of plasmid DNA (Sigma-Aldrich)

Serial No.	Sequence accession	Sequence (5' – 3')	Tm (ºC)
1	NFIA_027390 - F	GCCG**GAATTC**ATGCAGAACTTGTTCTTGTCCT	62
2	NFIA_027390 - R	AATTA**GAATTC**TTACCAACCTCTTCTAACTCTG	62
3	Afu6g12010 - F	GCCG**GAATTC**ATGGTTAGAAACTTGAAGCCAG	62
4	Afu6g12010 -R	AATTA**GAATTC**TTATCTACCGGACCAAACCAAA	62
5	ANID_05290.1 - F	GCCG**GAATTC**CGCACTGATCCACTCGTTCT	60
6	ANID_05290.1 - R	AATTA**GAATTC**TAGCAAACACGCAGTAGCCA	60
7	ANID_07327 - F	GCCG**GAATTC**GTATGTGGGTGCAACATGGC	60
8	ANID_07327 - R	AATTA**GAATTC**CCACCCACAAATCCTGACCA	60

*EcoR*I restriction sites highlighted in red. F: Forward primer; R: Reverse primer

6.2.5 Polymerase Chain Reaction

The general conditions for Polymerase chain reactions (PCR) were carried out as described by Green and Sambrook, 2012 (outlined in Table 6.3 below).

Table 6.3: PCR conditions for the amplification of hydrophobin genes from *A. nidulans*, and β-glucosidase genes from *N. fischeri*, *G. xylinus* and *A. fumigatus*

Accession No.	Initial denaturation step	Denaturation	Annealing temperature	Extension	No of cycles	Final elongation step
ANID_05290.1	95ºC, 5 min	95ºC, 1 min	55ºC, 2 min	72ºC, 2 min	30	72ºC, 5 min
ANID_07327	95ºC, 5 min	95ºC, 1 min	55ºC, 2 min	72ºC, 2 min	30	72ºC, 5 min
*AP012159.1	95ºC, 5 min	95ºC, 1 min	58ºC, 2 min	72ºC, 2 min	30	72ºC, 5 min
NFIA_027390	95ºC, 5 min	95ºC, 1 min	57ºC, 2 min	72ºC, 2 min	30	72ºC, 5 min
Afu6g12010	95ºC, 5 min	95ºC, 1 min	57ºC, 2 min	72ºC, 2 min	30	72ºC, 5 min

**G. xylinus*

Unigene model FTGENE2U thermocycler was used to amplify DNA/pDNA. PCR Master Mix (50 units/mL *Taq* polymerase, pH 8.5; 400µM of each dNTP; 3mM $MgCl_2$) was ordered from Promega (http://www.promega.com). The PCR was performed in a 50µL reaction containing 25µL Master Mix and varying volumes—and consequently, varying concentrations—of forward and reverse primers, genomic DNA and nuclease-free water for the purpose of comparison. Table 6.4 below summarizes the protocol.

Table 6.4: Component of PCR mixture for the amplification of hydrophobin and β-glucosidase gene

Component	Volume	Final concentration
Master Mix (2x)	25µL	1X
Forward primer (10µM)	0.5, 5.0µL	0.1, 1.0µM
Reverse primer (10µM)	0.5, 5.0µL	0.1, 1.0µM
DNA template (1000, 10000 fold dilutions)	5.0µL	< 250ng
Nuclease-free water	To make up 50.0µL	

Master Mix (Promega) contains *Taq* polymerase, dNTPs and $MgCl_2$. DNA-free reactions were also included to serve as negative controls in order to ensure that reagents were not contaminated with DNA.

6.2.6 Gel electrophoresis

The analysis of DNA extraction and PCR products was carried out using agarose gel electrophoresis. For genomic DNA extraction, 0.7% agarose in Tris-borate-EDTA (TBE) buffer (containing 0.45M Tris base, 0.45M Boric acid, and 0.02M EDTA with pH adjusted to 8.0 using 1M NaOH) was used while 1.2% agarose was used for PCR products, as they were expected to be smaller in size. Agarose gels were prepared by dissolving the appropriate amount of agarose in 100 ml TBE buffer, then heated in a microwave and allowed to cool to a temperature of ≈50^{o}C before adding 0.5µL web green, a nucleic acid dye that stains DNA to aid its visualization under UV light (Syngene Geneflow - serial No.: SYGV/4688). The gel was immediately poured into a gel tray and allowed to solidify. The tray was placed in an electrophoresis tank and covered with TBE buffer. 10µL of DNA preparation or PCR product was mixed with 2µL 6X loading buffer and the sample was loaded into the wells flanked by appropriate size markers. DNA preparation gels were run for 1 hour at 85V while PCR products were run for 4 hours at 39V and in some cases, the gel was run overnight at 15V.

Purification of amplified gene fragments from agarose gels was carried out using the QiagenMinElute^{R} Gel Extraction Kit (2011) following the manufacturer's instructions. The gel purified DNA was re-amplified using the PCR technique and the same primers. The product was examined using agarose gel electrophoresis as described above. The samples were always electrophoresed beside an appropriate molecular weight marker and the gel images were digitally captured.

6.2.7 Recovery of DNA from the gel

Purification of DNA from agarose gel was carried out using the QiagenMinElute^{R} Gel Extraction Kit (2011) following the manufacturer's instructions. The DNA fragments from the agarose gel were excised with a clean sharp scalpel. The gel slice was weighed in a colorless tube and 3 volumes of Buffer QG were added to 1 volume of gel (100 mg gel ~ 100 µl). This was incubated at 50^{o}C for 10 min (or until the gel slice had completely dissolved). The tube was vortexed every 2 – 3 min during incubation to help dissolve the gel. One gel volume of isopropanol was added to the sample and mixed by inverting. The sample was applied to the MinElute spin column placed in a provided 2 ml collection tube and centrifuged for 1 min. The flow-through was discarded and the MinElute spin column placed back into the same collection tube. 500 µl Buffer QG (Solubilization and buffer with pH indicator, 5.5 M guanidine thiocyanate [GuSCN], 20 mMTrisHCl pH 6.6) was added to the MinElute column and centrifuged for 1 min. The flow-through was discarded and the column placed back into the same collection tube. 750 µl Buffer PE (Wash buffer 10 mMTris-HCl pH 7.5, 80% ethanol) was added to the MinElute column and centrifuged for 1 min. The flow-through was discarded and the column placed back into the same collection tube. The column was centrifuged again for 1 min to completely remove residual ethanol from Buffer PE. To elute DNA, each MinElute column was placed into a clean 1.5 ml microcentrifuge tube and 10 µl Buffer EB (Elution buffer - 1 mM EDTA, 10 mM Tris.Cl, pH 8.5) was added directly onto the center of the MinElute membrane for

complete elution of bound DNA. The column was allowed to stand for 1 min and then centrifuged for 1 min. The purified DNA was analyzed on a gel by adding 1 volume of loading dye to 5 volumes of purified DNA.

6.2.8 Purification of PCR product

Purification of PCR products was carried out using the Qiagen QIAquick^{R} PCR Purification Kit (2011) following the manufacturer's instructions. 5 volumes of Buffer PB (Binding buffer which provides the optimal salt concentration and pH for adsorption of DNA to the QIAquick membrane - 30% isopropanol, 5 M Gu-HCl) was added to 1 volume of the PCR reaction and mixed. The mixture was applied to a QIAquick column placed in a 2 ml collection tube provided and centrifuged for 30 – 60 sec. The flow-through was discarded and the QIAquick column was placed back in the same tube. The column was centrifuged once more for 1 min to remove residual wash buffer. To elute DNA, the QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and 50 µl Buffer EB (10 mM Tris.Cl, pH 8.5) was added to the centre of the column membrane and allowed to stand for 1 min. The column was centrifuged for 1 min. The purified PCR product was examined using agarose gel electrophoresis as described above. The sample was electrophoresed beside an appropriate molecular weight. The gel images were digitally captured.

6.2.9 Cloning of PCR products

The purified PCR products were cloned into pGEM^{R}-T Easy vector systems (Appendix 37) following the manufacturer's instructions.

6.2.10 Optimization of ligation reactions

Control insert DNA and pGEM^{R}-T Easy vector was briefly centrifuged to collect contents at the bottom of the tubes. The ligation reaction was first optimized by setting up 2 similar ligation reactions using control insert DNA and 2X rapid ligation buffer (60 mM Tris-HCl, 20 mM $MgCl_2$, 20 mM Dithiothreitol, 2 mM ATP, pH 7.8) from Promega (Table 6.5). The

reactions were mixed by pipetting; one reaction was incubated at room temperature for 4 hours and the second reaction was incubated overnight at 4^{o}C. Two µl of each ligation reaction was added into a sterile 1.5 ml tube on ice for transformation.

Table 6.5: Optimization of ligation reactions using control insert DNA that came with the cloning kits from Promega and cloning into pGEM-T Vector

Reagents	Positive control (µl)	Background control (µl)
2X Rapid ligation buffer	5	5
pGEM^{R}-T Easy vector (50 ng)	1	1
Control insert DNA (4 ng/µl)	2	-
T4 DNA ligase (3 Weiss units/µl)	1	1
Nuclease free water	1	3
Final volume	10	10

6.2.11 Transformation of JM109 competent cells

Transformation of ligation reactions was carried out using *E. coli* JM109 high efficiency competent cells using the quick protocol from Promega with slight modifications. 50 µl of competent cells were supplemented with 2 µl of the ligation reaction. After transferring the competent cells into the ligation reaction, it was incubated on ice for 1 hour instead of 20 min before thermal heat shock. The heat shock of the cells was carried out for 2 min instead of 45 – 50 sec in the protocol, while incubation after adding Super Optimal broth with Catabolite repression (SOC) medium, was carried out at 150 rpm for 1 hour as against 1.5 hours in the protocol. Four volumes (300, 300, 200 and 200 µl) of each transformation solution were plated onto LB ampicillin/IPTG/X-Gal plates separately. Plates were

incubated at 37°C overnight and positive transformants cells were selected. After incubation the plates were stored at 4°C.

6.2.12 Ligation/transformation reaction

Ligation reactions were carried out as described in Table 6.6.

Table 6.6: Ligation reactions protocol of PCR products into pGEM-T Easy Vector

Reagents	Standard reaction (µl)	Positive control (µl)	Background control (µl)
2X Rapid ligation buffer	5	5	5
pGEM^{R}-T Easy vector (50 ng)	1	1	1
PCR product	1	-	-
Control insert DNA (4 ng/µl)	-	2	-
T4 DNA ligase (3 Weiss units/µl)	1	1	1
Nuclease free water	2	1	3
Final volume	10	10	10

Two µl of each ligation reaction was added into a sterile 1.5 ml tube on ice for transformation with slight modification from the manufacturer's instructions as described in section 6.2.11. A control tube with uncut Afu6g12010 was also prepared. Four volumes (300, 300, 200 and 200 µl) of each transformation solution were plated onto LB ampicillin/IPTG/X-Gal plates separately and plates were incubated at 37°C overnight. After incubation the plates were stored at 4°C.

6.2.13 Enzymatic restriction

Restriction enzyme digests were set up in a volume of 20 µl in a sterile tube in the order as described in the protocol (Table 6.7) below.

Table 6.7: Protocol for restriction enzyme digest of plasmid DNA and vectors

Component	Volume (µl)
Sterile, deionized water	11
Restriction enzyme 10X buffer	2.0
Plasmid DNA/Vector	6.0
*EcoR*I (10 unit/µl)	1.0
Final volume	20

The mixture was mixed gently by pipetting and centrifuged for a few seconds in a microcentrifuge. The obtained mixture was incubated for 4 hours at 37^{o}C and *EcoR*I was heat inactivated at 65^{o}C for 20 minutes.

6.2.14 Dephosphorylation reaction

Dephosphorylation reactions were carried out as described on Table 6.8 to prevent self-ligation of vector. Vector was dephosphorylated by addition of 1 µl calf intestinal phosphatase (CIP) and incubated at 37^{o}C for 60 minutes. Phosphatase was heat inactivated at 75 ^{o}C for 10 minutes before ligation with insert. After digestion, the different sized fragments were separated on a 1.2% agarose gel, gel extracted and purified as described in section 6.2.7 and the sizes determined by comparison with known DNA marker. The gel was visualized and the gel images were digitally captured under UV light.

Table 6.8: Protocol for dephosphorylation reaction of vectors

Component	Volume (µl)
Sterile deionized H_2O	10
CIP 10X buffer	2
Vector	7
Phosphatase	1
Final volume	20

6.2.15 Ligation

Digested DNA fragments were mixed with linearized vector in the presence of T4 DNA ligase from Invitrogen. The ligation reaction was set up as described in section 6.2.12 and products were used for transformation into *E. coli* JM109 cells to test the integrity and amplification of the construct.

6.2.16 Screening for positive transformants and plasmid purification of cloned fragments

Single white bacterial colonies were selected and inoculated into 20 ml sterilized LB broth containing suitable antibiotics (ampicillin – 100µg/ml or Zeocin – 25 µg/ml) and were grown overnight at 37^{o}C in an orbital shaker (Model G25, 390534557 U/K) at 150 rpm. Plasmid DNA was isolated for analysis using Qiagen^{R} plasmid plus midi kit (Cat. No. 12943) following the manufacturer's instructions. Bacterial cultures were harvested by centrifuging at 6000 x g for 15 min at 4^{o}C. The pelleted bacteria were completely re-suspended in 2 ml buffer P1 (Re-suspension buffer - 50 mMTris-HCl pH 8.0, 10 mM EDTA, and 100 µg/ml RNaseA). 2 ml buffer P2 (Lysis buffer - 200 mM NaOH, 1% SDS) was added and gently mixed by inverting until the lysate appeared viscous, and was incubated at room temperature (20^{o}C) for 3 min. QIAfilter cartridge was placed into a new and suitable tube allowing space for the addition of buffer BB (Binding buffer). 2 ml buffer

S3 was added to the lysate and mixed by inverting 4 – 6 times. The lysate was transferred to the QIAfilter cartridge and incubated at room temperature for 10 min. During incubation, Qiagen plasmid plus spin columns were placed into the QIAvac 24 plus and tube extenders were inserted into each column. The plunger was gently inserted into the QIAfilter cartridge and the cell lysate was filtered into the tube. 2 ml buffer BB was added to the cleared lysate and mixed by inverting 4 – 6 times. The lysate was transferred to a Qiagen plasmid plus spin column until the liquid had been drawn through all columns. To wash the DNA, 0.7 ml buffer ETR (Wash buffer - composition confidential to Qiagen but functioning as an endotoxin removal) was added and vacuum was applied until the liquid was drawn through all columns. To further wash the DNA, 0.7 ml buffer PE was added and vacuum applied until the liquid had been drawn through all columns. To completely remove the residual wash buffer, the column was centrifuged at 10,000 x g (9,700 rpm) for 1 min in a table top micro centrifuge. To elute the DNA, the Qiagen plasmid plus spin column was placed into a clean 1.5 ml tube, 200 µl buffer EB (elution buffer) was added at the centre and allowed to stand for ≥1 min and was later centrifuged for 1 min.

Plasmid DNA was amplified by PCR using appropriate primers to verify the integrity of the insert.

6.2.17 Glycerol stock of clones

Glycerol stocks of the bacterial clones were made for long-term storage. For storage of the clones, 0.85 ml aliquots of cells (in duplicates) from overnight cultures were mixed with 0.5 ml sterile 15% glycerol and stored at -20 and -80^{o}C.

6.2.18 Sequencing and analysis of positive clones

Sequencing of plasmid insert DNA was carried out at Source BioScience Life Sciences laboratory in Rochdale United Kingdom using forward primers (as described in Table 2.5) for each pDNA.

The sequences were read using Chromas Lite 2.1.1 sequence converter (Free Technelysium Software package for DNA sequencing – http://technelysium.com.au/?page_id=13). The sequence reads of appropriate quality obtained for each gene were compared against the non-redundant nucleotide sequence collection at NCBI Genbank using the web interface of NCBI-BLAST. EMBOSS (2000) Sixpack (http://www.ebi.ac.uk/Tools/st/emboss_sixpack/) and NADV (http://nadv.herokuapp.com/sequence/new_results) were used to display DNA along with translation in all 6 reading frames.

6.3 Results

6.3.1 Genomic DNA isolation and PCR amplification of ANID_05290.1 and ANID_07327

The genomic DNA extraction from *A. nidulans* using the method of Oakley lab and its isopropanol precipitation was successful (Figure 6.1) yielding high molecular weight bands larger than 21 kb which were used as template for PCR reactions.

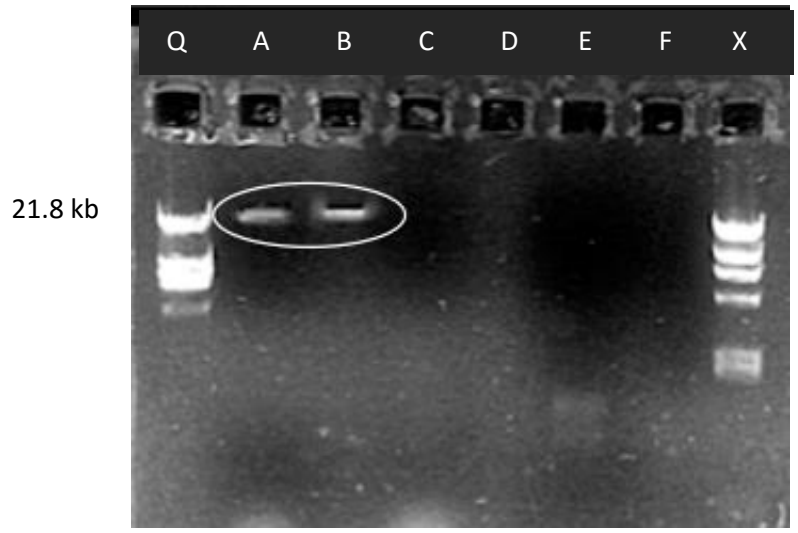


Figure 6.1: Electrophoresis analysis of the different DNA extraction methods from *A. nidulans*. DNA extraction Methods A, C and E are the extraction methods of Oakley lab, Liu *et al*., (2011) and Xu and Hamer (1995) respectively while methods B, D and F are their respective purified methods using isopropanol precipitation. The Oakley lab methods yielded high molecular weight bands. Q: λ *EcoR* I ladder, X: λ *Hind* III ladder.

PCR amplification for ANID_05290.1 and ANID_07327 was successful. The PCR products were electrophoresed beside an appropriate molecular weight ladder and the images were digitally captured. Figure 6.2 shows the gel electrophoretic result of the PCR product of ANID_05290.1 with a clear band of estimated size of 600 bp, which corresponds to the expected PCR product size of 618 bp.

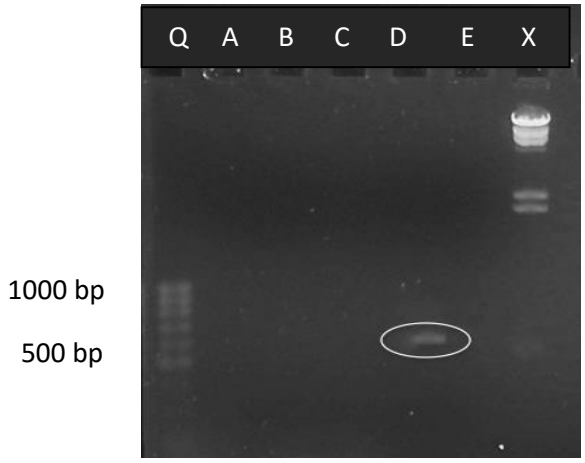


Figure 6.2: PCR product of ANID_05290.1 gene from *A.* nidulans. Q: PCR ranger ladder, A: Control DNA-free, B: 0.1 μM primers, 10^{-3} dilution of template DNA, C: 0.1 μM primers, 10^{-4} dilution of template DNA, D: 1 μM primers, 10^{-3} dilution of template DNA, E: 1 μM primers, 10^{-4} dilution of template DNA, X: λ *Hind* III

These same working conditions were also used to amplify gene ANID_07327. The results as shown in Figure 6.3 produced a band in lane C (1 μM of primers, 10^{-3} dilution of template DNA), which is estimated to be > 700 base pairs. This again corresponds to the expected size of the PCR product (794 base pairs).

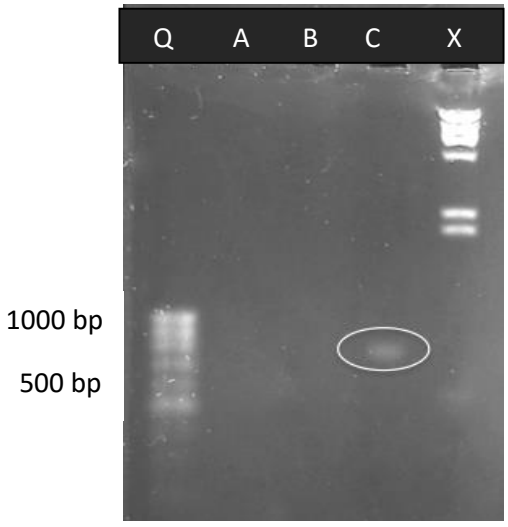


Figure 6.3: PCR product of ANID_07327 gene from *A. nidulans.* Q: PCR ranger ladder, A: DNA-free control, B: 0.1 μM primers, 10^{-3} dilution of template DNA, C: 1 μM primers, 10^{-3} dilution of template DNA, X: λ *Hind* III

Amplicons obtained were gel extracted as described in section 6.2.7 and fragments were purified. Gel purified products (Figure 6.4) were clear and were then used for ligation reactions.

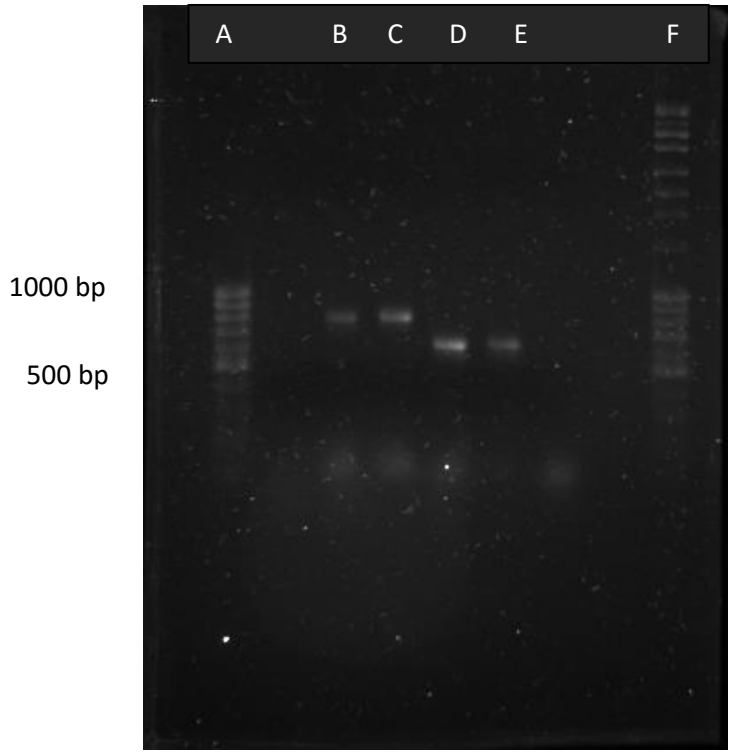


Figure 6.4: Electrophoresis gel images of ANID_05290.1 and ANID_07327 PCR products of gel purified hydrophobin genes. A: PCR product ladder, B and C: ANID_07327 gene, D and E: ANID_05290.1 gene, F: High ranger ladder

6.3.2 Optimization of ligation reaction using control insert DNA

Table 6.9 and 6.10 indicate results of the optimization studies using a control insert DNA from Promega. The incubation of ligation reaction overnight at 4^{o}C worked well. This ligation condition was chosen for subsequent ligation reactions as the ligation conditions yielded a large number of positive (white) clones. Purified PCR products of hydrophobin sequences were cloned into the pGEM-T Easy vector (Promega).

Table 6.9: Colony counting of transformation of control insert after 4 hours incubation of ligation reaction at room temperature

Sample	White colonies	Blue colonies
Positive Control (with 8 ng control insert DNA)	21	0
Background control (without insert DNA)	0	3

Table 6.10: Colony counting of transformation of control insert after overnight incubation of ligation reaction at 4ºC

Sample	White colonies	Blue colonies
Positive Control (with 8 ng control insert DNA)	43	15
Background control (without insert DNA)	0	2

6.3.3 Cloning and sequencing of ANID_05290.1 and ANID_07327 gene

PCR amplicons of ANID_05290.1 and ANID_07327 genes were gel purified and cloned as described in section 6.2.7. Figure 6.5C and Figure 6.5D showed *E. coli* transformants of hydrophobin ANID_05290 and ANID_07327 genes respectively selected on ampicillin plates.

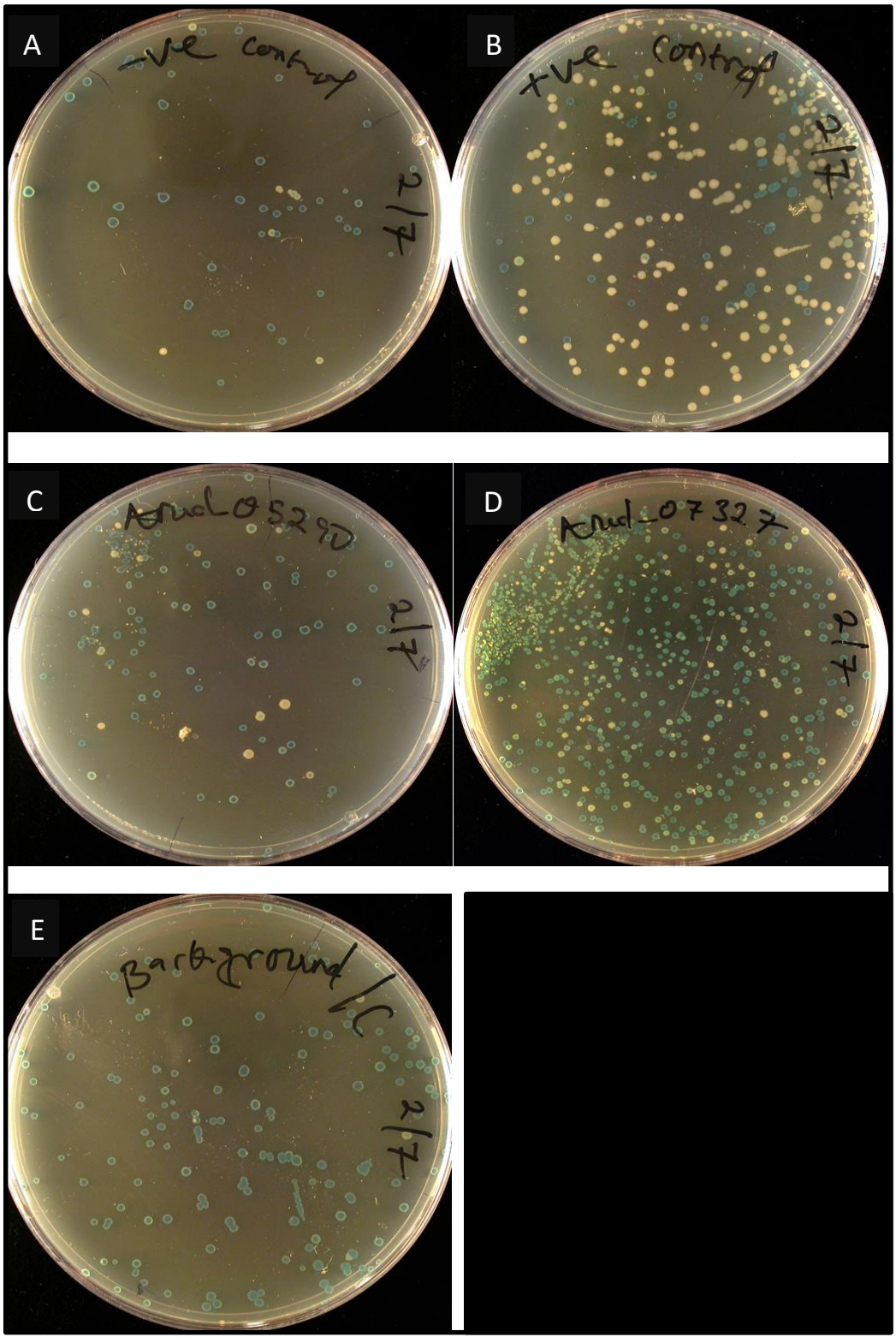


Figure 6.5: *E.coli* transformants of hydrophobin genes from *A. nidulans* selected on ampicillin plates. Potential clones are represented by white colonies where the *lacZ* reporter gene has been disrupted. A: Negative Control (Vector only); B: Positive Control (Vector + control insert + ligase); C: ANID_05290 clones; D: ANID_07327 clones; E: Background Control (Vector + ligase).

Approximately 14 and 50 clones were obtained for ANID_05290.1 and ANID_07327 respectively (Table 6.11). Amplification of an appropriate pDNA fragment by PCR was conducted in one colony of the respective clones in order to confirm the presence of the right insert.

Table 6.11: Colony counting of transformation of hydrophobin PCR products

Sample	White colonies	Blue colonies
PCR product (ANID_07327)	50	112
PCR product (ANID_05290.1)	14	70
Positive Control (with 8 ng control insert DNA)	214	38
Background control (without insert DNA)	2	106
Negative control	4	21

Note: The counts presented are from plate with 300 µl transformed cell solution. Transformation solutions were plated onto LB ampicillin/IPTG/X-Gal plates.

Plasmids were isolated from transformants and were purified using a Qiagen^{R} plasmid plus midi kit as described in section 6.2.16. The plasmid DNA (Figure 6.6) was prepared from a cultured cell originating from the colony and it was diluted to 10^{-1}, 10^{-2} and 10^{-3} fold, and used as a template DNA for PCR using same primers used for amplifying genomic DNA. In Figure 6.6, lane F and G are the plasmid DNA of *G. xylinum* (AP012159.1) (explained in details in Chapter 5, section 5.3.7).

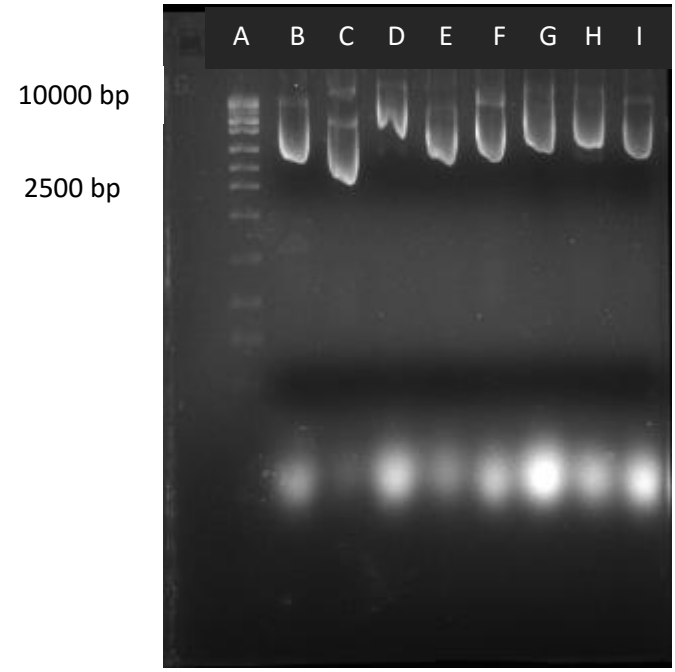


Figure 6.6: Electrophoresis gel images of ANID_05290.1, ANID_07327 pDNA and AP012159.1 Plasmid DNA. Lane A: High ranger ladder, B and C: ANID_05290.1 pDNA, D and E: ANID_07327 pDNA, F and G: *G. xylinum* (AP012159.1) β-glucosidase pDNA, H and I: positive control (Vector + control insert + ligase).

Figure 6.7 showed the digitally captured gel images of ANID_07327 and ANID_05290.1 colony PCR products. ANID_07327 and ANID_05290.1 plasmid contained an insert with approximately 700 bp and 600 bp respectively (Figure 6.7). The presence of amplicons with identical size from the genomic DNA confirmed the likely successful cloning of the respective hydrophobin genes from *A. nidulans* into pGEM-T vector.

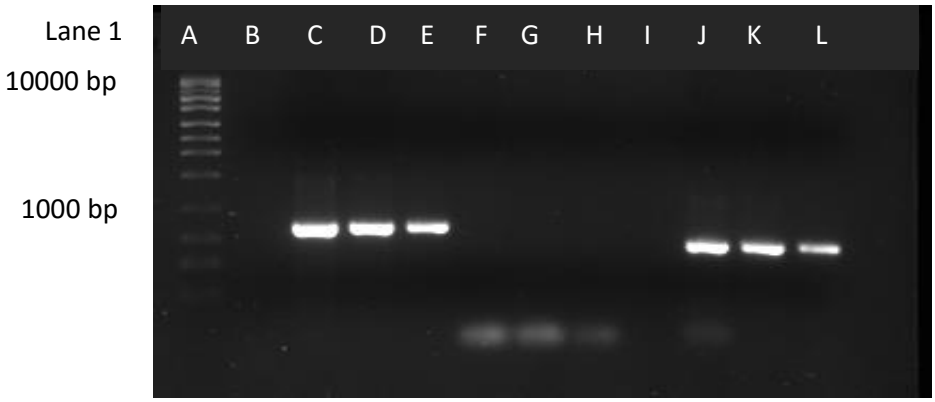


Figure 6.7: Electrophoresis analysis of presumed ANID_07327 and ANID_05290 of plasmid DNA PCR products. Lane 1; A: High range ladder, B: ANID_07327 negative control (no DNA), C, D and E- ANID_07327 (10^{-1}, 10^{-2}, 10^{-3}– pDNA dilutions), F, G and H – ANID_07327 hydrophobin pDNA (10^{-1}, 10^{-2}, 10^{-3} dilutions), I: ANID_05290.1 negative control (no DNA), J, K and L: ANID_05290.1 (10^{-1}, 10^{-2}, 10^{-3} pDNA dilutions).

6.3.4 Sequencing and BLAST analysis of ANID_05290.1 and ANID_07327 genes

Presumptive cloned genes were sent to Source BioScience LifeSciences and Eurofins Genomics for sequencing using the same forward primers for ANID_05290.1 and ANID_07327 (described in Table 6.1) for PCR amplification. The sequencing results confirmed integration of the recombinant gene into the pGEM-T vector, ANID_05290.1 and ANID_07327 insert returned 549 bp and 707 bp of sequence respectively (Figure 6.8 and 6.9).

BLASTn analysis of ANID_05290.1 sequence (Figure 6.8) indicated that the sequence has 99% identity with 90% coverage to *Aspergillus nidulans* FGSC A4 hypothetical protein with accession number AN5290.2 (XM_657802).The sequence also had close similarity with *Aspergillus nidulans* FGSC A4 chromosomeV (BN001305.1). The results indicated the existence of hydrophobin ANID_05290.1 insert in the clone.

>ANID_05290.1

AGCACAATGGTCCGACTTCTGCCTCTCACTCTCACCCTCGGCGCCTCTGCACTGGCAACCGAGC
GCGTTCAACTCCTTTCCCGCCAGGTAAGCGACTGCTCGCAGGTAAGCTATGTCGACTGCGGGAG
TAAATGCATGCCACCTGGCAACAGCTGTTGCGGGGATGGCATCTACTGTCGACCAGGCACCTAC
TGCGTCGTTAACGGCTGCTGCCCGATCGGTGAAGTTTGCACCGGGCCTGGGGGCACCATTACCG
AATGGTTCGACGTCACTACTACAGCGACCCTGACCGGCACTACCACCGTTACCGACGATGTTGA
GCCCACTGAAGCGCCGCCGGAGGACTCCACTACCACTGCCACCTCGTCTACCAGCTCACGGCCG
GGTATCCCCACGTCGAGCGAGTCCCAGCCGACCCAGTCGCCGACCGAGTCTACTCCGGTGCCTC
CTGTCTTCACCGGCGGCCAATCTGGCTTGCGCCCGGGCGTTGGTGCTGTTGCAGGCCTTATCGCT
GGAGCCGTCTTGCTATAATGGTTG

Figure 6.8: Nucleotide sequence of ANID_05290.1 matching sequence from Figure 6.12

BLASTx analysis of ANID_05290.1 sequence showed that the sequence has 98% identity with 70% coverage to *Aspergillus nidulans* FGSC A4 accession number AN5290.2 (XP_662894.1) with an E value of $3e^{-35}$.

BLASTn analysis of ANID_07327 sequence (Figure 6.9) indicated that the sequence has 99% identity with 75% coverage to *Aspergillus nidulans* FGSC A4 accession number AN7327.2 (XM_675504).The sequence also had close similarity with *Aspergillus nidulans* FGSC A4 chromosome IV (BN001304.1), *Salpingoeca sp* ATCC 50818 hypothetical protein (XM_0049882) and *Petromyzonmarinus neurotrophin* mRNA (EU449949.1). The results indicated the existence of hydrophobin ANID_07327 insert in the clone.

>ANID_07327

AATCGTCTGAAATTATCATCTAGCAAAAAACATTCCAACCAACACACTTGAGCAGCTATAGTGT
CTATTAGAAGTGGTAATCTGAGACTCTACTTCCGCCCACAATACTATCGACTATCTATCAAGCCC
GTTGACACTCGATAATCGGCACTACCAATCAAAATGCGGCTCACCTCGATCCCAATCGCCCTCC
TTCTCTCCCCTCTAGCTCTTGCAACGCCAAACCTCGTCGCAGAGAGCAACAATTCTATCCTTAAG
CGCAGCCCGTCACCCATGCGGCCACTCGTCGCTCGCCAGTCCTCGGCGTATATTCTGGCCCGCTC
TCCCCAGGACGACGATGGAGTGGTCTGCGACAGCGACGAGAAACGATGCGGCAATGCCTGTGT
AAATGAAGACTACAATGTTGTCCAGACAATGCAAACGGCGGGTGTCCGAGCGACGAAGAATGT
CAGAGGGATAACGGGGTCTGGGGGTGTTGTCCTGAGGGCGAGGACTGTCGCTGGGATGATGAC
GACGACGACGATGATAGGAATATCTTCGATAGGATCGGGGATGGAATTGATGATATTGGCGAT
GAGATCGAGGATGGATGGAACGATATCGTGAATGACGATGACGATGATGCGGCAGGGATGTTG
AAGCCCGGTTCGTTGCGTGATGGCGGCTGTCGTGGCGNCAGTGTTGCCGGCGTAGGTCGCTGGG

Figure 6.9: Nucleotide sequence of ANID_07327 matching sequence from Figure 6.12

BLASTx analysis of ANID_07327 sequence showed that the sequence has 75% identity with 39% coverage to *Aspergillus nidulans* FGSC A4 accession number XP_680596.1 with an E value of $5e^{-32}$.

6.3.5 Enzymatic restriction of NFIA_027390 β-glucosidase gene

The next step in the cloning strategy was an attempt to sub clone synthetic constructs of β-glucosidase and hydrophobin genes into expression vectors. The restriction enzyme digestion of the NFIA_027390 gene was carried out as described in section 6.2.13. Figure 6.10 showed the *EcoR*I restriction digest of NFIA_027390 pDNA. Electrophoresis results illustrated that the vector carrying the NFIA_027390 gene was fully digested and gave two bands of almost the same size which were overlapped. Bands overlapped because of the fact that the size of the pMK-RQ (Kan^R) vector and the NFIA_027390 insert are almost the same (2278 bp for the vector and 2232 bp for the insert).

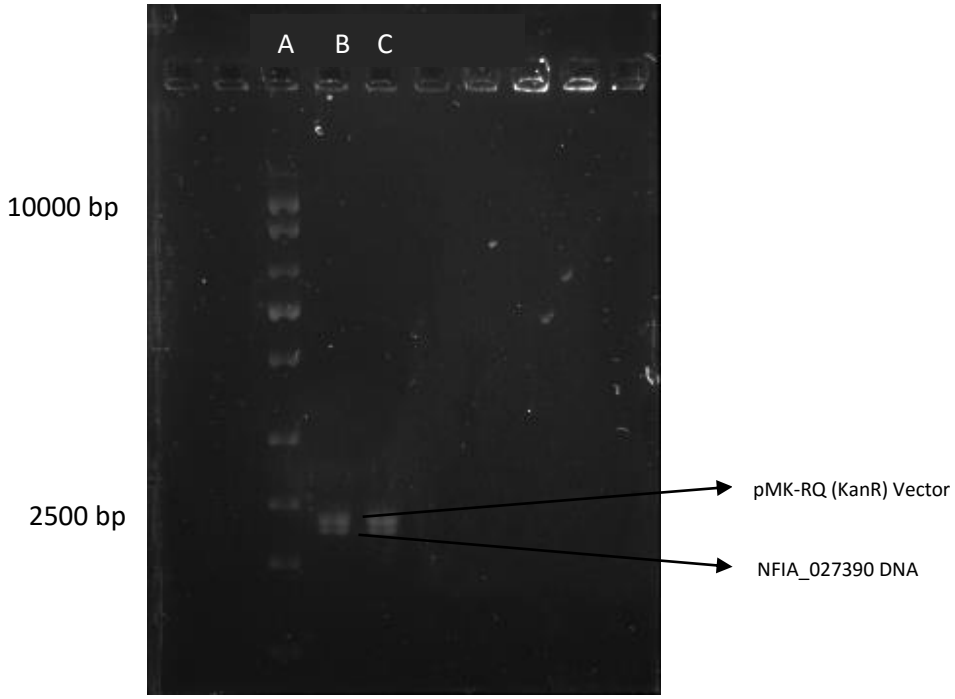


Figure 6.10: Electrophoresis analysis of the digested synthetic NFIA_027390 pDNA with *EcoR* I. A: High ranger ladder; B and C: NFIA_027390 DNA.

The plasmid map of pMK-RQ (Kan^R) carrying the synthetic NFIA_027390 β-glucosidase gene is illustrated in Appendix 29. Because of the overlap, it was difficult extracting the different bands and this prompted the design of specific primers flanked with *EcoR*I restriction sites to lift out the NFIA_027390, Afu6g12010, ANID_0529.1 and ANID_07327 genes. The primers are described in Table 6.2.

The NFIA_027390, ANID_0529.1 and ANID_07327 pDNA were amplified with primers flanked by *EcoR*I restriction sites using PCR. The PCR products were examined using agarose gel electrophoresis. The samples were electrophoresed beside an appropriate molecular weight maker.

The PCR products' gel electrophoresis result of the NFIA_027390 gene (Figure 6.11) showed a clear band of estimated size of 2200 bp base pairs, which corresponded to the product size of 2232 bp from the company where the artificial gene was synthesized.

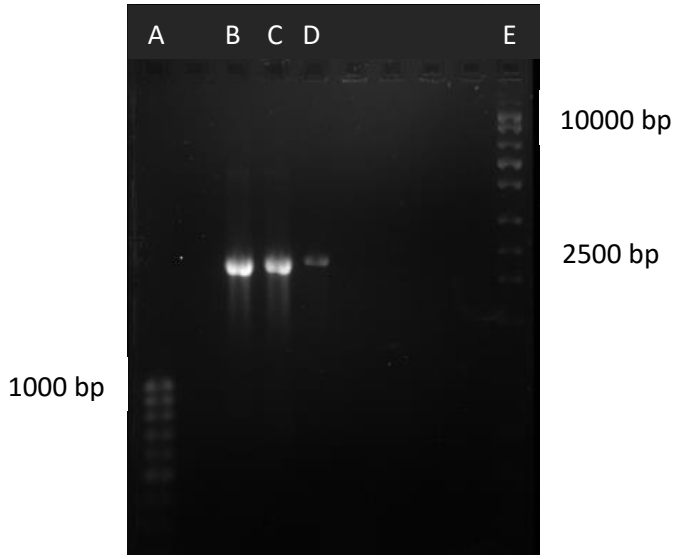


Figure 6.11: Electrophoresis analysis of NFIA_027390 PCR products flanked by *Eco*RI restriction sites. A: PCR product ladder, B, C and D: NFIA_027390 PCR products, E: High ranger ladder

The PCR gel electrophoretic result of ANID_0529 and ANID_073270 (Figure 6.12) also showed clear bands of estimated size of 600 bp and 700 bp, which corresponded to the expected PCR product sizes of 618 bp and 700 bp respectively

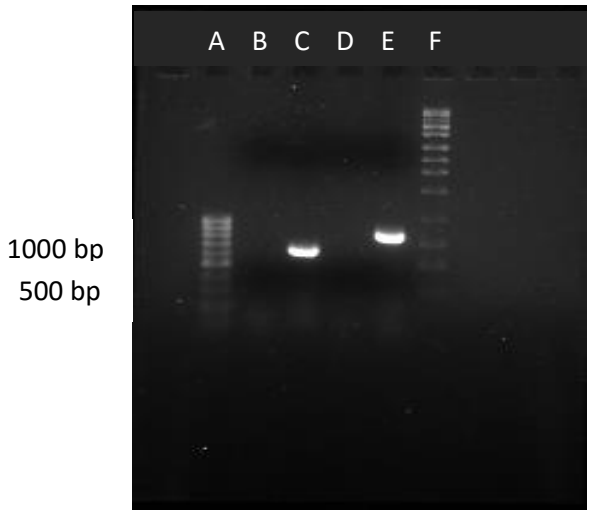


Figure 6.12: Electrophoresis analysis of ANID_0529.1 PCR products flanked by *Eco*RI restriction sites. A: PCR product size ladder, B: ANID_0529.1 negative control, C: ANID_0529.1 PCR product; D: ANID_073270 negative control, E: ANID_073270 PCR product; F: High ranger ladder

The PCR products of the amplified NFIA_027390 gene were further purified as described in section 6.2.8 and the product was digested with *EcoR*I and used for the subsequent cloning procedure.

6.3.6 Enzyme restriction of pPICZ A, pPICZ B and pPICZ C vectors

Figure 6.13 shows the electrophoresis gel images of the digested pPICZ A, pPICZ B, pPICZ C expression vectors with *EcoR*I. The results showed a clear band with an estimated size of 3300 bp which corresponds to the respective sizes of 3329 bp (pPICZ A), 3328 bp (pPICZ B) and 3329 bp (pPICZ C) of the vectors. The vectors were treated with alkaline phosphatase as described in section 6.2.14 to avoid re-circularization of vector.

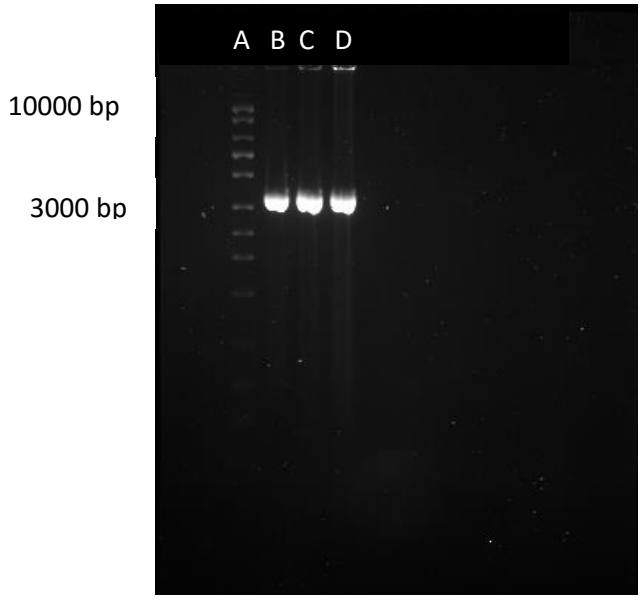


Figure 6.13: Electrophoresis analysis of the digested pPICZ vectors with *EcoR*I. A: High ranger ladder, B: pPICZ A, C: pPICZ B, D: pPICZ C. Samples were treated with alkaline phosphatase before electrophoresis.

6.3.7 Ligation of NFIA_027390 gene into pPICZ A

After enzyme restriction digest with *EcoR*I, the cleaved NFIA_027390 gene product was combined with the linearized pPICZ A vector in the presence of T4 DNA ligase. The

ligation and transformation reaction was set up as described in Table 6.12 following the manufacturers' instructions with the vector and the insert ratio of 1:1. The reaction was incubated at 4 ºC overnight and the recombinant expression vectors were transformed into *E. coli* JM109. 200 µl of each transformation solution were plated onto low salt LB zeocin plates and incubated at 37ºC overnight. After incubation the plates were stored at 4ºC.

Table 6.12: Ligation reactions for cloning NFIA_027390 PCR products into pPICZ A expression vector

Components	**Negative control (pPICZa CIPped, no ligase**	**Backgroung control (pPICZA CIPped, + ligase)**	**Positive control (pPICZA No CIP, + ligase)**	**Insert NFIA_027390**
5X T4 DNA ligase buffer	5	5	5	5
pPICZA vector	1	1	1	1
Insert	-	-	-	1
DNA ligase	-	1	1	1
Nuclease free H_2O	4	3	3	2
Total	10	10	10	10

6.3.8 Analysis of transformation of the expression vector into *E. coli* JM109

Selection of positive transformants were carried out on LB agar plates (low salt) containing 25 µg/ml zeocin. Figure 6.14 shows the *E.coli* transformants on zeocin plates. Approximately 82 potential clones were obtained.

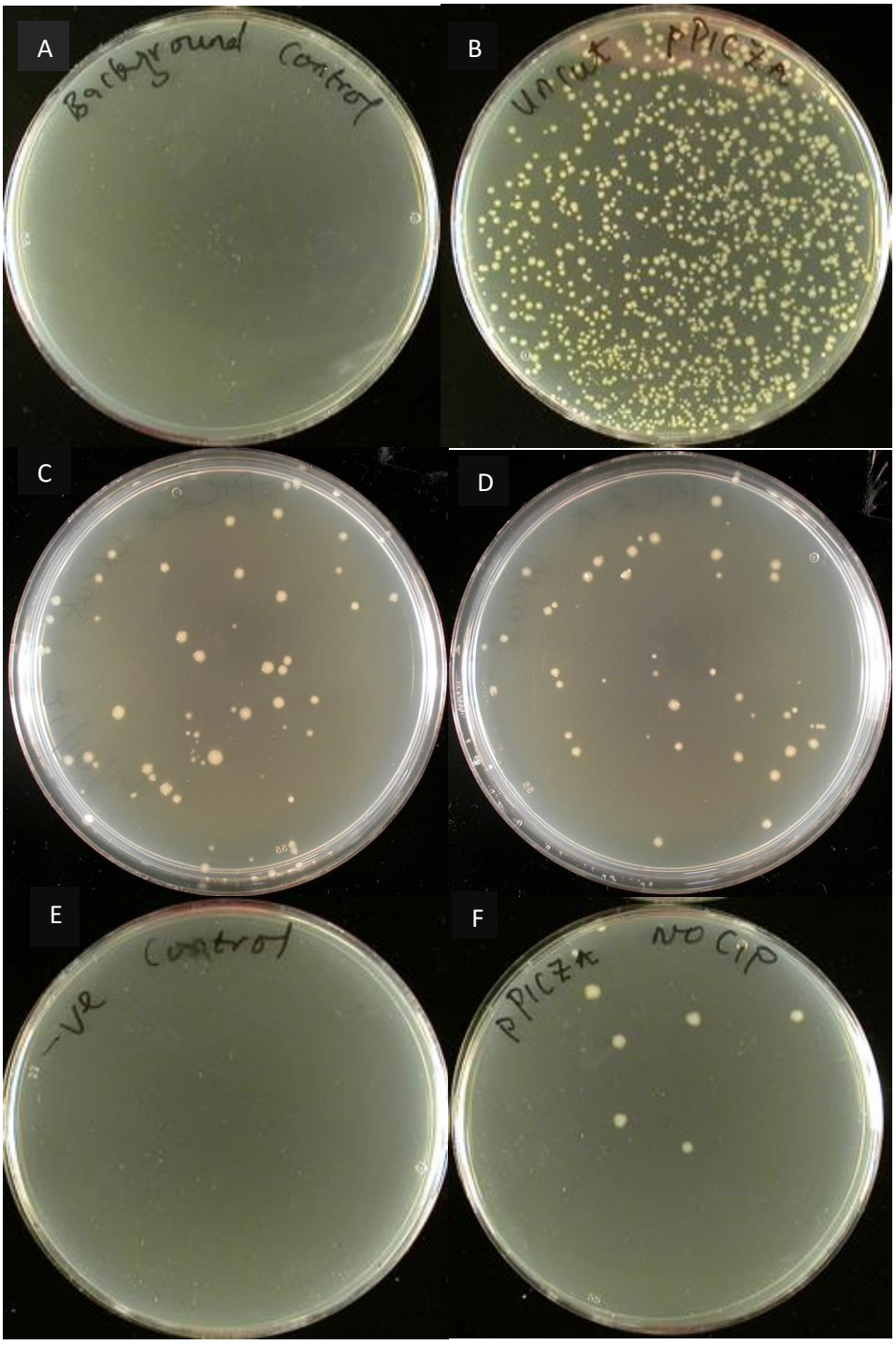


Figure 6.14: *E.coli* transformants of NFIA_027390 selected on zeocin plates. Potential clones are represented by white colonies in C and D. A: Background Control (pPICZ A vector CIPped, + ligase), B: Uncut pPICZ A expression Vector, C and D: NFIA_027390 Potential clones, E: Negative control (Restricted Vector CIPped, No ligase), F: Positive control (pPICZ A restricted vector not CIPped + ligase).

Ten zeocin resistant NFIA_027390 potential clones and one uncut pPICZ A clone were selected and inoculated into 5 ml sterilized low salt LB broth containing zeocin (25 µg/ml) and were grown overnight at 37ºC in an orbital shaker (Model G25, 390534557 U/K) at 150 rpm. Cells were harvested and the plasmid DNA was isolated for analysis using Qiagen^{R} plasmid plus midi kit (Cat. No. 12943) according to the manufacturer's instructions (section 6.2.16).

Figure 6.15 shows the electrophoresis pDNA gel images of the ten selected potential clones carrying NFIA_027390 gene. These plasmids clearly differed in mass indicating the likely presence of inserts. The NFIA_027390 pDNA on lane B, C, E, F, G, H and K were selected for colony PCR because the bands were sharp and were of different sizes.

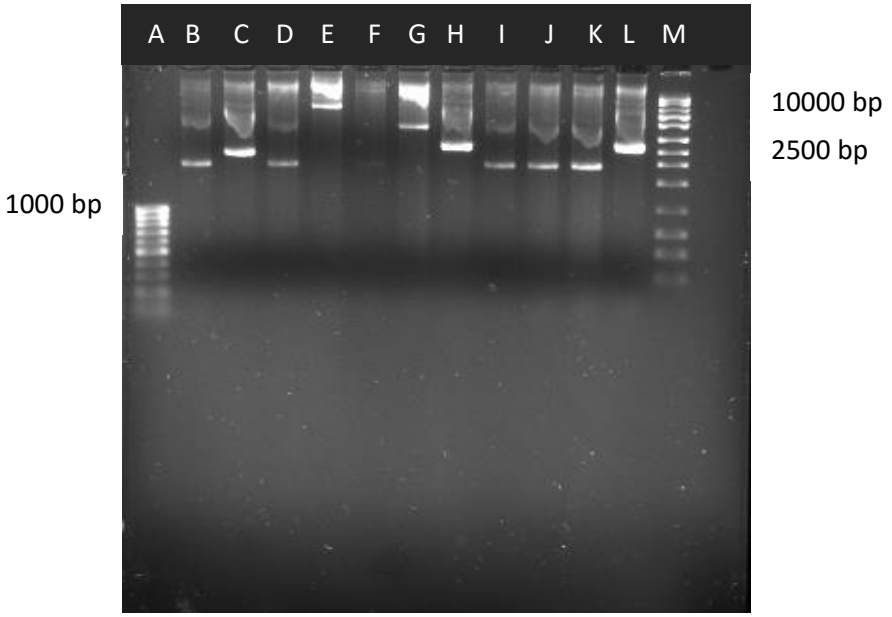


Figure 6.15: Electrophoresis gel images of NFIA_027390 plasmid DNA and Uncut pPICZ A pDNA. A: PCR product ladder, B, C, D, E, F, G, H, I, J and K: pDNA of NFIA_027390 from the ten different potential clones, L: Uncut pPICZ A clone, M: High ranger ladder

Colony PCR was carried out using the same primers (Table 6.2) that were used in the initial PCR amplification of NFIA_027390 gene. Colony PCR was carried out to verify the integration of the insert into pPICZ A.

Figure 6.16 shows the PCR electrophoresis gel images of the selected NFIA_027390 pDNA flanked by uncut NFIA pDNA which serve as a positive control. The gel images revealed that there was no PCR amplification of the selected pDNA from lane B, C, D, E, F, G and H. This therefore means that the NFIA_027390 gene was unlikely to be integrated into the pPICZ A vector.

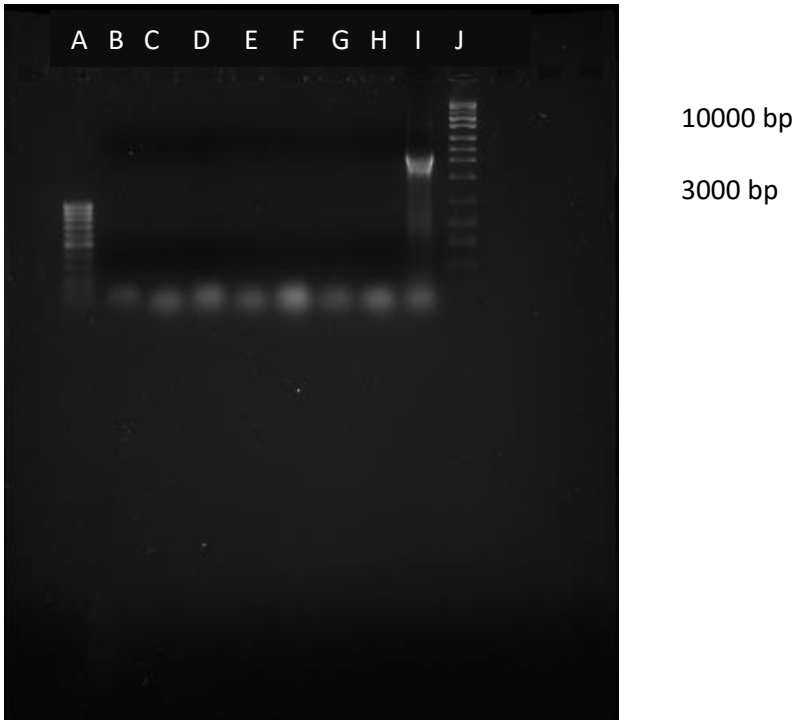


Figure 6.16: Electrophoresis gel images of NFIA_027390 pDNA PCR products. Lane A: PCR product ladder, B, C, D, E, F and G: NFIA_027390 pDNA PCR products, H: DNA free negative control, I: Positive control (Uncut NFIA pDNA from the Invitrogen company), J: High ranger ladder

A single zeocin resistant NFIA_027390 (ligated into pPICZ A) potential positive clone was further selected for pDNA extraction. Cleaved NFIA_027390 gene was also ligated into pPICZ B expression vector. After the transformation of the ligation products into *E. coli* JM109, selection of positive transformants was carried out on low salt LB agar. Figure 6.17 shows the electrophoresis gel image of plasmids from the NFIA_027390 gene ligated into pPICZ A and plasmids from NFIA_027390 gene ligated into pPICZ B with uncut

pPICZ B as a control. The result showed pDNA of NFIA_027390 ligated into pPICZ B with high molecular weight of approximately 8000 – 10,000 bp while NFIA_027390 pDNA in pPICZ A gave a molecular weight of approximately 2500 bp.

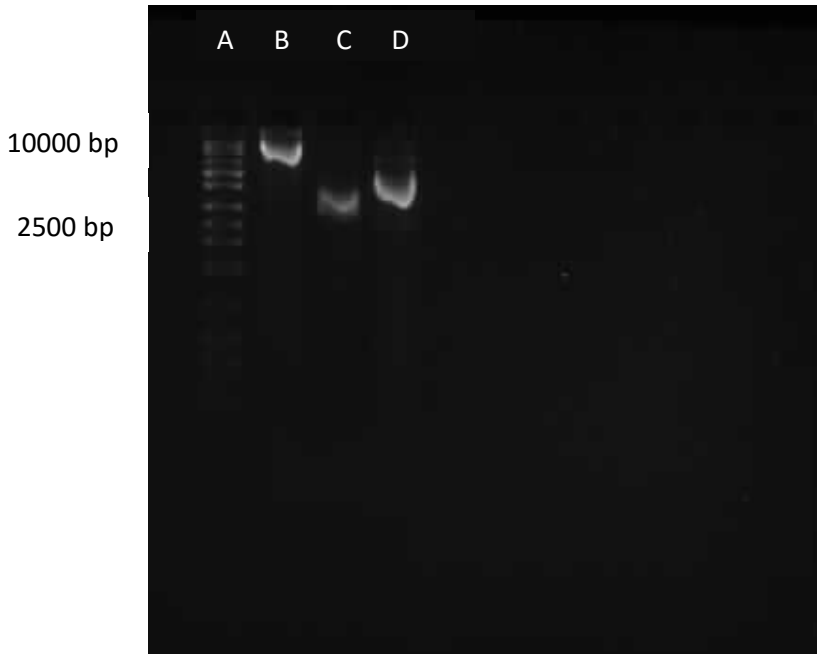


Figure 6.17: Electrophoresis gel images of NFIA_027390 pDNA ligated into pPICZ B. A: High ranger ladder, B: NFIA_027390 pDNA in pPICZ B, C: NFIA_027390 pDNA in pPICZ A, D: pPICZ B uncut pDNA

The pDNA were gel extracted and colony PCR was carried out using the same primers (Table 6.2). Figure 6.18 shows the electrophoresis gel images of the amplified NFIA_027390 genes. The pPICZ A plasmid contained an insert with approximately 2500 bp (Figure 6.18, lane F and G) while the pPICZ B plasmid also gave a faint amplicons of approximately 2500 bp (Figure 6.18, lane E). The amplicons were gel purified and sent to Source BioScience LifeSciences for sequencing to check the integration of the inserts into pPICZ A and pPICZ B expression vectors. Sequencing was carried out with *AOX* 5' primers (Invitrogen).

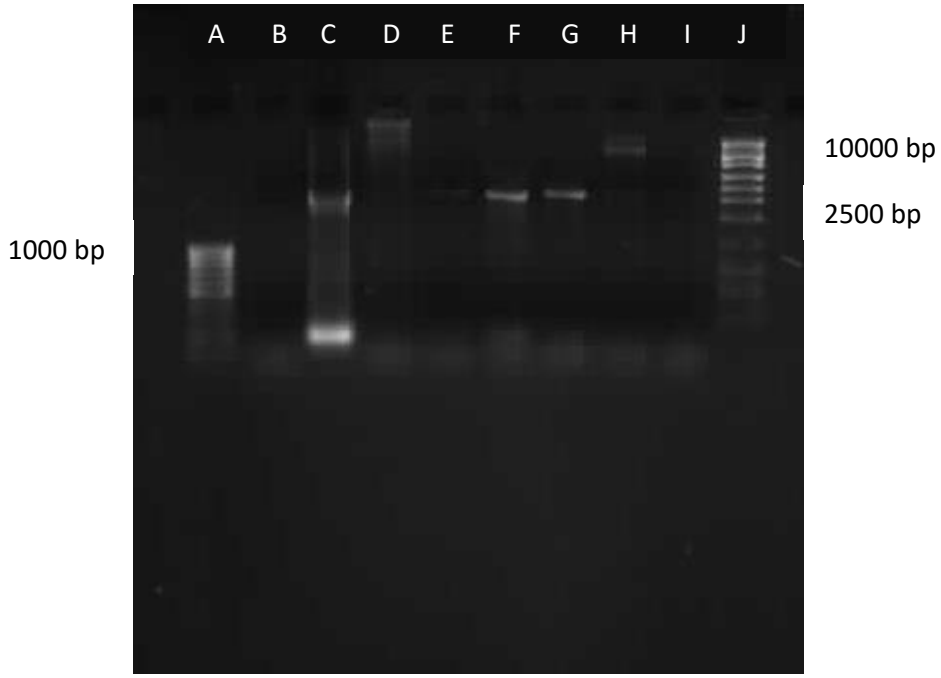


Figure 6.18: Electrophoresis gel images of pDNA PCR products of NFIA_027390. Lane A: PCR product ladder, B: Negative control (primers and master mix only), C: positive control (uncut NFIA_027390 pDNA), D and E: NFIA_027390 clone into pPICZ B (E is in 10^{-1} dilution), F and G: NFIA_027390 clone into pPICZ A (G is in 10^{-1} dilution), H and I: pPICZ B uncut plasmid (I is in 10^{-1} dilution), J: High ranger ladder.

BLASTn analysis of NFIA_027390A sequence indicated that the sequence has 99% identity with 82% coverage (E value = 0.0) to Expression vector LIC-pPICZ-LC2, complete sequence with accession number JF327851.1. The sequence also had 71% coverage and 100% identity similarity with Expression vector pPICZalphaH6E, complete sequence with accession number KM035419.1. BLASTn analysis did not indicate the presence of NFIA_027390 gene in pPICZ A plasmid. Manual search for NFIA_027390 gene in the pPICZ A plasmid indicated an empty vector without an insert (see Appendix 6).

BLASTn analysis of NFIA_027390B sequence also indicated that the sequence has 91% identity with 84% coverage (E value = 0.0) to Expression vector LIC-pPICZ-LC2, complete sequence with accession number JF327851.1. The sequence also had 91% identity and 75% coverage similarity (E value = 0.0) with Expression vector

pPICZalphaH6E, complete sequence with accession number KM035419.1. BLASTn analysis did not also indicate the presence of NFIA_027390 gene in pPICZ B plasmid. Manual search for NFIA_027390 gene in the pPICZ B plasmid indicated an empty vector without an insert (see Appendix 7).

At this stage, further attempts at sub cloning into expression vectors were abandoned owing to time constraints.

6.3.9 Cloning and sequencing of ANRA12.6 and ANRA12.9 gene

Two β-glucosidase genes from *A. niger* F321 denoted as ANRA12.6 and ANRA12.9 were amplified using PCR techniques with two set of primers. The primers are described on Table 6.1. The PCR products of ANRA12.6 and ANRA12.9 gave estimated PCR products of 1,190 bp and 1,950 bp respectively, while the colony PCR of their pDNA gave estimated sizes of 860 bp and 1,600 bp respectively.

Presumptive cloned genes were sent to Source BioScience LifeSciences (UK) using the same forward primers for ANRA12.6 and ANRA12.9 (described in Table 6.1) for PCR amplification. The sequencing results confirmed integration of the recombinant gene into the pGEM-T vector (Figure 6.19 and Figure 6.20).

BLASTx analysis of ANRA12.6 sequence (Figure 6.19) indicated that the sequence has 99% identity with 84% coverage to *Aspergillus niger* 513.88 (accession number - XP_001394592.2) β-glucosidase protein with an E-value of $1e^{-97}$. The sequence also had close similarity with *A. niger* ATCC1015 hypothetical protein (accession number - EHA19734.1) with 99% identity, 84% coverage and E-value of $1e^{-96}$. The results indicated the existence of ANRA12.6 insert in the clone.

> ANRA12.6

GGAAAGCAGGCTACTCCCTAACCGACTCCTCTCGTCATCCTCCCCAAGTGGACACCTC
CCCTACACCATCCCCTACAAGGAATCCGACTACCCATCCAGCGTGGGCCTCCTCGACC
AAGCCTTCGGACAAATCCAAGACGATTTCACCGAGGGCCTATACATCGACTACCGCCA
CTTCCTCAAAGCCAACATCACTCCCCGCTACCCGTTTGGTCACGGCCTCTCCTACACCA
CCTTCACCTACTCCACTCCATCCCTCACCACCGTCACCGACCTCGACACTGCCTACCCC
CCAGCTCGCTCATCCAAAGGCCCAACCCCATCGTACAGCACCGCCATCCCAAACCCAT
CCGAGGTCGCCTGGCCATCCTCCTTCTCTCGTATCTGGCGCTACTTGTACCCATATCTC
GACAACCCGCAATCTGTCACCAACTCCTCTTCATCTTCATACCCCTATCCATCCGGCTA
CTCCACCACTCCGAAACCTGCACCCCGAGCTGGTGGTGGCGCCGGTGGAAATCCCGCC
CTGTGGGATGTTGCCTTCGCGGTGGACGTAACCATCACAAACAGTGGGAACGTTAGCG
GTCGCGCTGTGGCCCAGCTGTATGTCGAGCTACCGACCGATACACTGGGGGTAGATGC
GCCATCCAGACAGTTACGGTAGTTTGAGAAAACGGGGATCCTGGAGCCCGGGAGAGT
CAGACGGTGGTGCTGAATGTTACGAGGAAGGATGTGAGTTTTATGTTTTCCTTTTTTTT
TTTTTTTT

Figure 6.19: Nucleotide sequence of ANRA12.6

BLASTx analysis of ANRA12.9 sequence (Figure 6.20) indicated that the sequence has 96% identity with 93% coverage to *Aspergillus niger* ATCC 1015 (accession number – EHA19734.1) hypothetical protein with an E-value of 0.0; and also with β-glucosidase from *Aspergillus kawachii* IFO 4308 (accession number – GAA83698.1) with identity of 95%, coverage of 93% and E-value of 0.0. The result suggests the existence of ANRA12.9 insert in the clone.

Figure 6.21 shows the open reading frame (ORF) using JavaScript DNA translator 1.1 (Perry, 2002) of ANRA12.6 (frame 1) and ANRA12.9 (frame 3).

> ANRA12.9

CCTCCGCTCTGCCTTGCTCGCGACGCCATCACCCTTCTCAAGAACGAAAATAATACTCT
CCCTTTGTCGACAACCGACTCACTAAAGATCTTTGGCACTGACGCCGGCCCCAACTCC
AACGGGCTCAACTCGTGCACCGACCAAGGCTGCGACAACGGCGTCCTCACCATGGGCT
GGGGCAGTGGCACGTCTCGTCTCCCATATCTTGTAACCCCCCAACAAGCCATCGCCAA
TCTCTCTTCCTCCGCAGAATTCTACATCACCGACTCATTCCCGTCCGACCTCAACCCCA
ATGCCTCTGACATCGCGCTCGTCTTCATCAACGCCGACTCCGGCGAAAACTACATCAC
CGTCGAAAACAACCCCGGTGACCGCACCACCGCCGACCTCTACGCGTGGCACAACGG
CGACGATCTCGTCAAAGCCGCAGCAGACGTCTTCTCCACCGTCATCGTGATTGTCCAC
ACCGTCGGCCCTATTCTCCTCGAGAATTGGATTGATTTGGACCCCGTCAAAGCCGTCCT
CATCGCCCATCTCCCCGGTCAAGAAGCAGGCTACTCCCTAACCGACATCCTCTTCGGCT
CATCCTCCCCAAGTGGACACCTCCCCTACACCATCCCCTACAAGGAATCCGACTACCC
ATCCAGCGTGGGCCTCCTCGACCAAGCCTTCGGACAAATCCAAGACGATTTCACCGAG
GGCCTATACATCGACTACCGCCACTTCCTCAAAGCCAACATCACTCCCCGCTACCCGTT
TGGTCGCGGCCTCTCCTACACCACCTTCACCTACTCCACTCCATCCCTCACCACCGTCA
CCGACCTCGACACTGCCTACCCCCCAGCTCGCTCATCCAAAGGCCCAACCCCATCGTA
CAGCACCGCCATCCCAAACCCATCCGAGGTCGCCTGGCCATCCTCCTTCTCTCGTATCT
GGCGCTACTTGTACCCATATCTCGACAACCCGCAATCTGTCACCAACTCCTCTTCATCT
TCATACCCCTATCCATCCGGCTACTCCACCACTCCGAAACCTGCACCCCGAGCTGGTG
GTGGCGCCGGTGGAAATCCCGCCCTGTGGGTTGCCTTCGCGGTGGACGTAACCATCAC
AAACAGTGGAACGTTAGCGCGCGCTGGCCCAGCTGTATGTCGAGCTACCGACCGATCA
CTGGGGTAGATGCGCATCCAACTTACGGCATTTGAAAGGGCCTCCGGGATCA

Figure 6.20: Nucleotide sequence of ANRA12.9

>ANRA12.6 frame1

GKQATPPTPLVILPKWTPPLHHPLQGIRLPIQRGPPRPSLRTNPRRFHRGPIHRLPPLPQSQHHSPLPVW
SRPLLHHLHLLHSIPHHRHRPRHCLPPSSLIQRPNPIVQHRHPKPIRGRLAILLLSYLALLVPISRQPAIC
HQLLFIFIPLSIRLLHHSETCTPSWWWRRWKSRPVGCCLRGGRNHHKQWERRSRCGPAVCRATDRY
TGGRCAIQTVTVVENGDPGARESQTVVLNVTRKDVSFMFSFFFFF

>ANRA12.9 frame3

SALPCSRRHHPSQERKYSPFVDNRLTKDLWHRRPQLQRAQLVHRPRLRQRRPHHGLGQWHVSSPIS
CNPPTSHRQSLFLRRILHHRLIPVRPQPQCLHRARLHQRRLRRKLHHRRKQPRPHHRRPLRVAQRRR
SRQSRSRRLLHRHRDCPHRRPYSPRELDFGPRQSRPHRPSPRSRSRLLPNRHPLRLILPKWTPPLHHPL
QGIRLPIQRGPPRPSLRTNPRRFHRGPIHRLPPLPQSQHHSPLPVWSRPLLHHLHLLHSIPHHRHRPRH
CLPPSSLIQRPNPIVQHRHPKPIRGRLAILLLSYLALLVPISRQPAICHQLLFIFIPLSIRLLHHSETCTPSW
WWRRWKSRPVGCLRGGRNHHKQWNVSARWPSCMSSYRPITGVDAHPTYGIKGLRD

Figure 6.21: Deduced amino acid sequences of ANRA12.6 and ANRA12.9 (Perry, 2002)

Figure 6.22 shows the multiple sequence alignment of β-glucosidases from *Aspergillus niger* AnBg11, ANRA12.6 and ANRA12.9. A complete sequence alignment was carried out on the alignment program TCoffee (Notredame *et al*., 2000) to generate a multiple sequence alignment. The results as indicated in Figure 6.22 shows core catalytic active sites of ANRA12.6 and ANRA12.9 not to be aligned with those of AnBgl1 as described by Lima *et al*., (2013). The carbohydrate-binding PA-14 domain (Yoshida *et al*., 2010) was absent in both ANRA12.6 and ANRA12.9 sequences. Also, the N-terminal domain, which is suggested to act as solubility enhancers for the folding C-terminal domains *in vivo* (Kim *et al*., 2007), was missing in ANRA12.6.

Figure 6.22: Multiple sequence alignment of β-glucosidases amino acids from *Aspergillus niger* AnBg11, ANRA12.6 and ANRA12.9. Symbols: Catalytic site (★); helix –A and –B (green box), intermediate amino acids from linker 2 and N-terminal domain (black boxes) (Lima *et al*., 2013).

Figure 6.23 shows the phylogenetic trees (neighbour joining) of β-glucosidase sequences were constructed using ClustalX and Treeview (Larkin *et al*., 2007) with the bootstrap value set at 2000. Phylogenetic tree was constructed to compare *Aspergillus* enzymes (AN12.6 and ANRA12.9) to bacterial counterparts (from *Gluconacetobacter xylinus, Bacillus* subtilis) and with *A. niger* (AnBg11), Afu6g12010 and NFIA_027390 enzymes. Characterized bacterial enzymes were included in tree drawing so as to root the trees in order to identify whether ANRA12.6 and ANRA12.9 enzymes resembled bacterial enzymes. However, it became clear that examples of bacterial β-glucosidases in some cases were grouped with ANRA12.6 and ANRA12.9 enzymes (Figure 6.23).

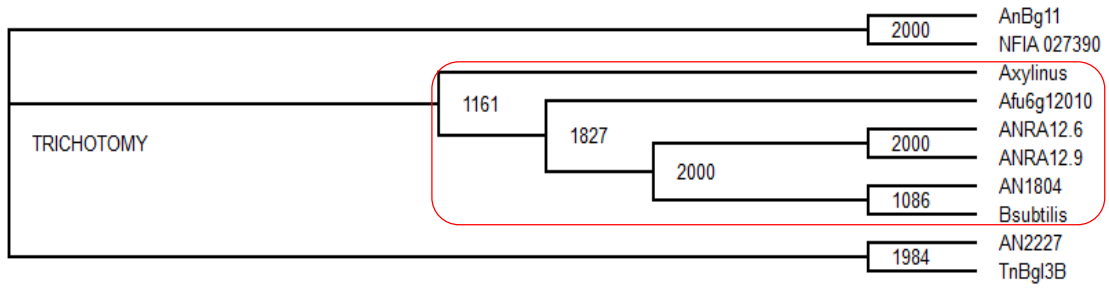


Figure 6.23: Treeview analysis of β-glucosidases: *A. niger* (AnBg11, ANRA12.6, ANRA12.9), *Thermotoga neapolitana* (TnBgl3B) NFIA_027390, *A. nidulans* (AN1804.2), and bacterial enzymes from *Gluconacetobacter xylinus* (*G. xylinus*) (WP_007399076) and *Bacillus subtilis* (YP_003864539.1) (shown with values out of 2000 bootstraps for branch points). Red rounded rectangle: an example of fungal and bacterial proteins forming a clade.

6.4 Discussion

6.4.1 Cloning and sequencing of ANID_05290.1 and ANID_07327 hydrophobin genes

ANID_05290.1 and ANID_07327 hydrophobin genes were successfully cloned into the pGEMT vector. Though these genes were initially not classified as encoding hydrophobins, Littlejohn *et al*., (2012) have classified these proteins as belonging to an intermediary class of hydrophobins. The ANID_05290.1 and ANID_07327 clones were sequenced and their sequence was searched for homologous sequence in GenBank. ANID_05290.1 and ANID_07327 genes were shown to have homology with other fungal hydrophobin genes. ANID_05290.1 had the highest homology (99% identity) to the hydrophobin XM_657802.1 (Galagan *et al*., 2005) while ANID_07327 showed highest homology (99%) identity to the hydrophobin XM_675504.1 (Galagan *et al*., 2005). The complete nucleotide sequences with deduced amino acid sequences for ANID_05290.1 and ANID_07327 clones are shown in Figure 6.24 and 6.25 respectively.

```
1      _____M__V__R__L__L__P__L__T__L__T__L__G__A__S__A__L__A__T__
1      AGCACAATGGTCCGACTTCTGCCTCTCACTCTCACCCTCGGCGCCTCTGCACTGGCAACC
19     E__R__V__Q__L__L__S__R__Q__V__S__D__C__S__Q__V__S__Y__V__D__
61     GAGCGCGTTCAACTCCTTTCCCGCCAGGTAAGCGACTGCTCGCAGGTAAGCTATGTCGAC
39     C__G__S__K__C__M__P__P__G__N__S__C__C__G__D__G__I__Y__C__R__
121    TGCGGGAGTAAATGCATGCCACCTGGCAACAGCTGTTGCGGGGATGGCATCTACTGTCGA
59     P__G__T__Y__C__V__V__N__G__C__C__P__I__G__E__V__C__T__G__P__
181    CCAGGCACCTACTGCGTCGTTAACGGCTGCTGCCCGATCGGTGAAGTTTGCACCGGGCCT
79     G__G__T__I__T__E__W__F__D__V__T__T__T__A__T__L__T__G__T__T__
241    GGGGGCACCATTACCGAATGGTTCGACGTCACTACTACAGCGACCCTGACCGGCACTACC
99     T__V__T__D__D__V__E__P__T__E__A__P__P__E__D__S__T__T__T__A__
301    ACCGTTACCGACGATGTTGAGCCCACTGAAGCGCCGCCGGAGGACTCCACTACCACTGCC
119    T__S__S__T__S__S__R__P__G__I__P__T__S__S__E__S__Q__P__T__Q__
361    ACCTCGTCTACCAGCTCACGGCCGGGTATCCCCACGTCGAGCGAGTCCCAGCCGACCCAG
139    S__P__T__E__S__T__P__V__P__P__V__F__T__G__G__Q__S__G__L__R__
421    TCGCCGACCGAGTCTACTCCGGTGCCTCCTGTCTTCACCGGCGGCCAATCTGGCTTGCGC
159    P__G__V__G__A__V__A__G__L__I__A__G__A__V__L__L__*________
481    CCGGGCGTTGGTGCTGTTGCAGGCCTTATCGCTGGAGCCGTCTTGCTATAATGGTTG
```

Figure 6.24: Nucleotide sequences and deduced amino acid sequences of ANID_05290.1

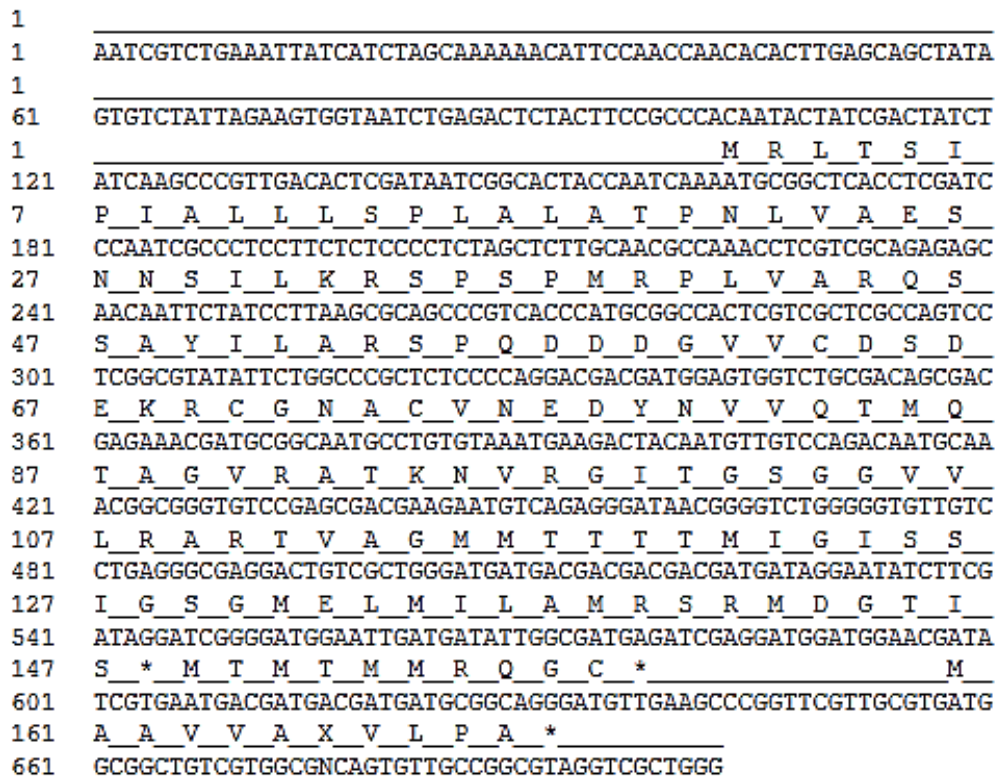


Figure 6.25: Nucleotide sequences and deduced amino acid sequences of ANID_07327

Cloned ANID_05290.1 encodes a protein of 158 amino acid residues while cloned ANID_07327 encodes a protein of 161 amino acid residues. The amino acid sequences of both ANID_05290.1 and ANID_07327 revealed that they do not fall into either the class I or the class II traditional classification of hydrophobins though they have the hydrophobin motif signature. This intermediate structure is particularly attractive for biotechnological applications as it may combine the attractive the features of both classes for ease of production and purification (Littlejohn *et al*., 2012).

6.4.2 Cloning and sequencing of NFIA_027390 β-glucosidase gene

The cloning and expression of β-glucosidases from fungal and bacterial sources has been reported by several authors (Karnaouri *et al*., 2013; Meko'o *et al*., 2010; Tajima *et al*.,

2001; Paavilainen *et al*., 1993). β-glucosidase is known for its ability in hydrolyzing cellobiose to fermentable sugars (Cox *et al*., 2000; Harhangi *et al*., 2002; Liu *et al*., 2012). In this study, the full length DNA of synthetic NFIA_027390 β-glucosidase gene was inserted into pPICZA and pPICZB vector and transformed into *E. coli* JM109 to give pPICZ-NFIA_027390 expression plasmid. Figure 6.14 C and D shows the potential clones in *E. coli* transformants selected on zeocin plates represented by white colonies. Confirmation of insertion was achieved by PCR (Figure 6.18 Lane D and E for pPICZB; Lane F and G for pPICZA). Appendix 6 and 7 also showed the potential nucleotide sequence of the NFIA_027390 clone into pPICZA and pPICZB respectively. A BLAST search against sequences deposited in the GenBank database revealed that the NFIA_027390 β-glucosidase gene was not inserted into the pPICZA and pPICZB vector thereby returning an empty vector.

What could have gone wrong? First is the problem of non-digested or partially digested plasmid. This problem could be as a result of inadequate restriction enzyme, too much DNA, failure to deliver reaction buffer or inadequate mixing of the enzyme into the DNA and the buffer. Secondly, it is possible that T4 Ligase is not working; this is because T4 Ligase is an unstable commercial enzyme. Slight contamination with DNA wash buffers or chemical inhibitor can affect ligation activity (BioLabs troubleshooting guide for cloning). Other factors that could be responsible for ligation failure could be religation of the cut vector with itself, phosphatase reaction was incomplete or the phosphatase was not working. Last but not the least; it could be that satellite colonies were selected for pDNA extraction (Manna *et al*., 2013).

6.4.3 Cloning and sequencing of ANRA12.6 and ANRA12.9 gene

A novel gene, ANRA12.6 and ANRA12.9 encoding β-glucosidases were cloned from *A. niger*. The β-glucosidases gene ANRA12.6 and ANRA12.9 were PCR amplified using

primer sequences found in closely related fungal species. The resultant amplicons were successfully cloned into pGEM-T vector. When the sequences were analysed by the SignalP 4.1 program (Petersen *et al*., 2011), it was predicted that the encoded ANRA12.6 and ANRA12.9 β-glucosidase enzyme could be secreted because it had a signal peptide. The open reading frame (ORF) using JavaScript DNA translator 1.1 (Perry, 2002) of ANRA12.6 (frame 1) and ANRA12.9 (frame 3) encoded a protein of 255 amino acids and 405 amino acids (Figure 6.21) with a predicted molecular mass of 29.23 kDa and 47.95 kDa respectively (http://www.bioinformatics.org/sms/prot_mw.html).

Figure 6.22 shows both ANRA12.6 and ANRA12.9 to have good alignment with the AnBgl1 which is a further confirmation of the BLAST analysis (section 6.3.9) that they are both fungal β-glucosidases. Figure 6.23 showed ANRA12.6 and ANRA12.9 proteins grouping with bacterial proteins (*G. xylinus and B. subtilis*) with over 50 % bootstrap value (red rounded rectangle). The grouping of ANRA12.6 and ANRA12.9 with the bacterial proteins could be as a result of convergent evolution where proteins have evolved from different species to fill the same role.

Generally, *Aspergillus* species are known as a useful source of β-glucosidases (Dan *et al*., 2000; Kamaruddin *et al*., 2015). Kamaruddin *et al*., (2015) have described a β-glucosidase from *A. niger* which is active at low pH values (pH 3.0 – 6.0) as well as high temperature (60 ^{o}C) and the enzyme effectively hydrolyses pNPG with a specific activity of 347.62 U/mg. Baba *et al*., (2015) also characterized *Aspergillus aculeatus* β-glucosidase 1 (AaBGL1) and compared its properties to a commercially supplied orthologue in *Aspergillus niger* (AnBGL). Their results showed recombinant AaBGL1 to be more insensitive to glucose inhibition and more efficient at hydrolyzing one of major transglycosylation products, gentiobiose than AnBGL. The recombinant AaBGL1 also completely hydrolysed 5 % cellobiose to glucose faster than AnBGL. In this study, a β-

glucosidase gene was successfully cloned from a novel *A. niger* strain thus achieving the first step towards developing recombinant β-glucosidases capable of degrading cellulosic material.

6.5 Conclusion

In this study, encoding sequences for two novel *A. nidulans* G0281hydrophobin genes and β-glucosidases from *A. niger* 321 were successfully cloned into pGEM-T vector. Bioinformatics studies suggested that both ANRA12.6 and ANRA12.9 β-glucosidases share some characteristics with the bacterial proteins.

Chapter 7

General discussion and conclusions

7.0 Introduction

Globally, 140 billion metric tons of biomass is generated every year from agriculture (UNEP, 2009). Nigeria potentially generates 168.49 million tonnes of biomass every year (Simonyan and Fasina, 2013). As raw material, biomass, such as wood chips, food waste, corn cobs etc. has an attractive potential as energy source in Nigeria, but it is still largely under-utilized and left to rot or openly burned in the fields. Most cities spend 20 – 50 % of their annual budget on waste management and only 20 – 80 % of the waste is collected (Ayuba *et al*., 2013); where the collection and transportation accounts for between 70-80% of total waste management cost (UNDP, 1998). The production of biofuel from agricultural waste and biomass has become a necessary goal for a clean and healthy environment particularly in developing countries (UNEP, 2009; Popp *et al*., 2014). Pettitt *et al*., (2010) described the Neter 30 in-vessel composter which was designed for composting organic wastes and the optional addition of horticultural wastes in order to provide biodegradable waste recycling systems. This process enables the diversion of approximately 20 tonnes of food waste (from kitchen and food preparation areas) from landfill and produces about 10 tonnes of compost per year. Nigeria could be a major player in the biofuel producing industry considering the amount of agricultural waste and biomass available. The present project describes one contribution to this approach by exploiting a range of cellulose degrading enzymes and associated proteins – hydrophobins.

Endoglucanases, exoglucanases and β-glucosidases are cellulase enzymes that work together in hydrolysing cellulose (Wang *et al*., 2011; Liu *et al*., 1996). β-glucosidase completes the final step in cellulose hydrolysis by converting cellobiose to glucose thereby circumventing the effect of feedback inhibition on endoglucanase and exoglucanase activity (Flannelly *et al*., 2015). In fungi such as *Trichoderma reesei*, β-glucosidase is produced in lower quantities e.g. 742 $U.L^{-1}$ (De Castro *et al*., 2010; Tiwari *et al*., 2013) while in *Aspergillus* species the β-glucosidase enzyme is produced in large quantities e.g.

514 I $U.g^{-1}$ (Kang *et al*., 1999; Junior *et al*., 2014). The β-glucosidase enzyme has attracted attention because whilst it represents a limiting factor in cellulose degradation being subject to feedback inhibition by glucose, some fungal strains still lack this enzyme which is widely used in lignocellulose degradation (Bai *et al*., 2013). Endoglucanases and exoglucanases synergistically work together with β-glucosidases in hydrolysing cellulose by attacking the cellulose material from both reducing and non-reducing ends. The importance of hydrophobins in the possible degradation of lignocellulose along with other lignocellulolitic enzymes is discussed in section 1.2.6 and section 3.1.2. In this study, two approaches were used to identify candidate β-glucosidase enzymes, the bioinformatics approach (Chapter 3) and plate assay methods coupled to a qualitative biochemical assay (Chapter 4).

7.1 Bioinformatics analysis of *Aspergillus* β-glucosidase and hydrophobin genes

β-glucosidases are glucosidase enzymes that act upon β-1→4 bonds linking two glucose and are the key enzyme components present in cellulase that complete the final step during cellulose hydrolysis by converting cellobiose to glucose (Singhania *et al*., 2013). In an effort to develop cost effective processes in producing β-glucosidases for cellulose degradation, the protein sequences of potential β-glucosidases from the *Aspergillus* genus were searched. A total of 166 proteins were identified as β-glucosidases after manual BLASTp search on *Aspergillus* comparative database; fifty one of which have been officially annotated while one hundred and fifteen are hypothetical. Inconsistency in the number of β-glucosidases was observed particularly in the *Aspergillus* genus when compared with the report of Coutinho *et al*., (2009) which could be as a result of a continual process of re-annotation and error correction of the genomic database. Evidence for Horizontal Gene Transfer (HGT) of bacterial origin of some β-glucosidases for example in *A. nidulans* AN1804.2 was provided by their lack of introns, absence of some fungal specific amino acid insertions in their primary protein sequence and unusual

positions in phylogenetic trees showing similarities to bacterial proteins (Chapter 3). The results, for example, highlighted the unexpected association of AN1804.2, Afu6g12010 and AO090005000337 with bacterial counterparts suggesting HGT events from bacteria to fungi (Chapter 3).

Horizontal gene transfer is the process of exchange of genetic material between distantly related species and is one of the most important evolutionary forces within microbial populations (Andersson, 2005; Baltrus, 2013). The horizontal movement of genetic material between distantly related organisms has been suggested to play an important role in the evolution of organisms by either increasing fitness in order to survive in complex and interesting environments, or allow the colonization of new environments (Schmitt and Lumbsch, 2009; Mallet *et al*., 2010). For example, bacteria normally produce a whole arsenal of chemical products known as polyketide groups which are used for signalling, defence and communication. Polyketides are also present in organisms that are sharing mutualistic relationships such as colonies of sponges and symbiotic bacteria (Gould, 2012). While many of the new fungal polyketides will have evolved within the fungi, it is also strongly suggested by Gould, (2012) that a group of them originally came from bacteria by HGT. Why might *Aspergillus* species have retained β-glucosidase genes acquired from bacteria? The spectacular thermo-tolerance and very broad pH profile (Figure 6.34) of AN1804.2 could be an indication of why the fungus has kept the bacterial gene. *Aspergillus* species are renowned for their ability to colonise a wide range of habitats of different pH values – an ability often attributed to the induction of extracellular enzymes appropriate to the ambient pH (Cray *et al*., 2013). Clearly an enzyme with a naturally broad pH profile would be an attractive addition to the proteome. Similarly a secreted enzyme with a high thermo-tolerance may allow the fungus to access substrates within decomposing material somewhat remote from the location of the producer organism. Previous studies have shown that whole bacterial genomes have been incorporated into

other organisms without large fitness consequences, while other studies have catalogued a library of phenotypic effects that potentially are by-products of HGT, including thermal tolerance (Baltrus, 2013; Epstein *et al*., 2014; Dougherty *et al*., 2014).

Applying a new HGT-Finder tool to the *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus nidulans* genomes, Nguyen *et al*., (2015) found 273, 542 and 715 transferred genes respectively. Schmitt and Lumbsch (2009) used comparative phylogenetics to infer the ancestor of a group of polyketide synthase genes in fungi. They aligned keto synthase domain sequences of all available fungal 6-methylsalicylic acid (6-MSA)-type PKSs and their closest bacterial relatives. Considering the large number of 6-MSA-type PKS sequences found in fungi, Schmitt and Lumbsch (2009) hypothesize that the evolution of typical lichen compounds was facilitated by the gain of polyketide synthase from bacteria. Studies on HGT in eukaryotes have also been published and documented by other authors (e.g. Gastebois *et al*., 2011; Coelho *et al*., 2013; Jaramillo *et al*., 2014; Ropars *et al*., 2015). Coelho *et al*., (2013) studied the phylogenetic distribution and pattern of inheritance of a fungal gene encoding a fructose transporter (*FSY*1) with unique substrate selectivity. In a survey that included 241 available fungal genomes, 109 *FSY*1 homologues were identified in two sub-phyla of Ascomycota and 10 independent inter-species instances of HGT involving *FSY*1 were identified. Nikolaidis *et al*., (2013) also reported a rare case of HGT in whole plant expansin genes that code for plant cell wall loosening protein. These were suggested to be transferred from plants to bacteria and fungi. The HGT of beta-lactam biosynthesis genes from bacteria to fungi has been the subject of discussion for many years. Unlike most other fungal genes, beta-lactam biosynthesis genes are clustered and some of these genes lack introns (Brakhage *et al*., 2009).

However, the mechanisms involved in DNA exchanges between distant species are mostly unknown, either between eukaryotes or to explain the numerous reports of horizontal transfers between prokaryotic and eukaryotic organisms (Mallet *et al*., 2010; Tan and

Tomkins, 2015). Given that β-glucosidases play an important role in cellulose degradation, it appears particularly promising to study β-glucosidase biosynthetic genes of bacterial origin in fungi. Commercial systems for cellulose degradation demand activity at low pH and thermo-tolerance, reflecting the need to pre-treat lignocellulose with acid at high temperature (Sørensen *et al*., 2013). The broad pH (Figure 4.15) and temperature (Figure 4.17) profiles of β-glucosidase preparation from AN1804.2 was successfully demonstrated, making this a potentially potent enzyme for efficient cellulose degradation.

7.2 Screening of fungal and bacterial strains for growth and cellulase production

Prior to the advent of the genome projects, the conventional way to detect β-glucosidase activity would be thorough enzyme assays. Precise quantitative assays (e.g. Onyike *et al*., 2008, Chapter 4) cannot process sufficiently large samples for initial screens, so leading to the development of cruder but rapid plate assays. The present work describes the application of a modified plate assay (Chapter 4). β-glucosidases play an important role in many biological processes and are widely distributed in nature. They are found in plants, humans and cellulolytic microorganisms such as bacteria and fungi (Tiwari *et al*., 2013). In humans, β-glucosidase enzyme is involved in the hydrolysis of glucosyl ceramides, the deficiency of which leads to Gautier's disease (Barton *et al*, 1990; Harzer and Yildiz, 2015). In plants, β-glucosidases are involved in ripening of fruits, pigment metabolism, defence mechanisms and β-glucan synthesis during plant cell wall development (Krisch *et al*., 2010; Tiwari *et al*., 2013); while in microorganisms (fungi and bacteria), the enzyme is involved in cellulose synthesis induction and hydrolysis (Tajima *et al*., 2001; Tiwari *et al*., 2013; Gao *et al*., 2013; Menendez *et al*., 2015). Cellulolytic fungi and bacteria can easily be grown in simple media and are reported to be the best choice for cellulase production because they produce cellulase extracellularly when grown on cellulose as a carbon source, and it is easy to separate the enzyme from the microbial biomass (Mandels and Weber, 1969; Gokhale *et al*., 1991; Sarkar and Aikat., 2014). In the present study, different fungal

and bacterial sources were screened for cellulolytic activity. Twenty one fungi and eight bacterial strains were selected for this study. The effect of pH, temperature and carbon sources was studied with an intention to suggest suitable conditions for the production of crude cellulase enzyme. These microbes showed considerable variation in their capacity to degrade cellulose (Appendix 31). Microorganisms are the most diverse and abundant group of living organisms that live in a wide variety of environments. Gans *et al*., (2005) estimated that 10 grams of unpolluted soil will contain 8.3 x 10^6 different species of bacteria. The diversity of fungal species present in a gram of soil has also been estimated to be in hundreds or thousands (Jumpponen and Jones, 2009). *Aspergillus niger* is one of the most important organisms which is widely used in biotechnology and fermentation industry and has been reported to grow over an extremely wide pH range (pH 1.4 – 9.8) with a relatively high temperature optimum at 35 – 37 ^{o}C (Schuster et al., 2002). *Aspergilli* are known to produce an extensive range of plant cell wall degrading enzymes and many species of the genus have been identified to possess all component of the cellulase complex (Ja'afaru, 2013). Reddy *et al*., (2014) isolated and screened fungi from the soil. Their results showed that fungal isolates had appreciable cellulose degradation properties and the highest cellulase producing isolates were *Aspergillus niger* and *Aspergillus flavus*; and the least was *Trichoderma sp*. Hu *et al*., (2011) co-cultivated *Aspergillus niger* and *Aspergillus oryzae* together on wheat bran. The results of their enzyme assay for plant polysaccharide and lignin-degrading enzymes showed that co-cultivation can improve extracellular enzyme production. Future studies in the screening of fungal and bacterial strains for extracellular cellulase enzyme production should include the co-cultivation of *Aspergillus niger* and *Aspergillus nidulans* with each other and with bacterial strains respectively.

The advantages of the conventional plate assay method used in this study (Chapter 4) is that it is rapid and inexpensive, and offers an obvious advantage over the genomic approaches where no genome data is available for novel organisms. A rapid plate assay

method will later be needed at a later stage in this project for screening potential clones where integration events govern different levels of expression (Ahmad *et al*., 2014). The main disadvantage of the rapid plate assay method is that it does not give a quantitative measurement of the sample and more freezer space is required to store prepared plates.

7.3 Production and degradation of bacterial cellulose, cloning and characterization of novel β-glucosidases from *G. xylinus*

Poor waste management practices, in particular widespread dumping of waste on major roads, highways, waterways, water bodies and uncontrolled dump sites generate the problem of low sanitation levels in major cities across Nigeria (see Appendix 39). Waste management infrastructure is almost non-existent particularly in slum areas that constitute a big part of many of the cities and towns (Mwesigye *et al*., 2009). As a result, there is the need for the government to support the production and recycling of BC supplemented plastic as pure plastics take a longer time to decompose. Supporting recycling will complement the existing structure and means of waste management in Nigeria (Imam *et al*., 2008). This study provides for the first time a fully detailed characterisation of pure bacterial cellulose before and after treatment with a commercially available cellulase enzyme (Chapter 5). The properties of the BC obtained in this study compared well with literature-cited publications (Castro *et al*., 2011; Qin *et al*., 2015; Atwa *et al*., 2015). Recent thermogravimetric analysis by Feng *et al*., (2015) showed that BC produced by *Gluconacetobacter hansenni* CGMCC 3917 has high thermal stability, indicating BC as a potential additive for packaging materials for its good mechanical properties and moisture retention ability. It has also been demonstrated that by-product streams from the biodiesel industry and waste streams from confectionary industries could be used as the sole sources of nutrients for the production of BC with similar properties as those produced with commercial sources of nutrients such as glucose and sucrose (Tsouko *et al*., 2015).

In addition, a novel β-glucosidase gene from *G. xylinus* was also successfully cloned and sequenced. The amplicons on sequencing confirmed homology of *G. xylinus* β-glucosidase sequence to *G. xylinus* 3288 (AP012159). Apart from Tajima *et al*., (2001) who cloned and sequenced a β-glucosidase gene from *G. xylinus* ATCC 23769, there is no literature readily available on the cloning and sequencing of β-glucosidase from *G. xylinus*. The use of commercial *Aspergillus* cellulase enzyme in the hydrolysis of BC in this present study suggests an attractive prospect for the production and recycling of biopolymers from bacterial sources.

Because of the advances in recombinant technology and protein engineering, enzymes from microbial sources have acquired much attention for industrial use and are preferred due to their economic feasibility, high yield, rapid growth of microbes on inexpensive media, ease of product modification and optimization, stability and greater catalytic activity (Gurung *et al*., 2013). Biomass degrading enzymes are usually produced in large scale by commercial companies using fungi or bacteria by feeding the microbes with agricultural residues (Balan, 2014). *Phanerochaete chrysosporium* is reported as one of the commercially cultivated fungi with varying abilities to utilize lignocellulosic materials (Pothiraj *et al*., 2006). As a result of poor waste management in Nigeria, the popular slogan of environmental issues in big cities in the recent times is "environmental sanitation" (Mshelia, 2015). Extremely large financial dedication is required to recruit and train enough manpower and also acquire materials for dealing with waste management as the rate of waste generated is far more than the rate of safe disposal (Mshelia, 2015). The enzymatic degradation of agricultural wastes (rice straw and cassava peels) in Nigeria using bacterial and fungal strains such as *Pseudomonas sp*., *Bacillus sp*., *Trichoderma viride* and *Aspergillus terreus* has been revealed by the work of Michael and Obasola (2015). The conversion of rice straw and cassava peels means that these agricultural wastes could be used as raw materials by industries. An attractive solution for biomass/waste

treatment in Nigeria is to treat some of these accumulated wastes with microorganisms that produce cellulase in a system like that described by Pettitt *et al*., (2010), using an in-vessel waste composter, so as to recycle and convert wastes into useful materials; thereby turning waste to wealth.

7.4 Cloning of hydrophobin genes, cloning and expression of β-glucosidases

In section 1.2.12, three hypotheses were put forth regarding how the *Aspergillus* genus harbours β-glucosidase genes with attractive properties; *G. xylinus* produces BC and β-glucosidases with novel characteristics; and how fungal β-glucosidases require co-production of proteins such as hydrophobins to allow access to lignocellulose (Delmas *et al*., 2012).

In this study, the encoding sequences for two novel *A. nidulans* hydrophobin genes that do not fall into either class I or class II category were successfully cloned (Chapter 6; Section 6.3.3). Class I hydrophobins are insoluble in aqueous solvents which makes them unsuitable in some applications while class II hydrophobins are soluble in aqueous solvents but show rapid rates of layer formation (Littlejohn *et al*., 2012). The discovery of ANID_05290 and ANID_07327 as an intermediary class of hydrophobins in *Aspergillus* has raised hopes that they may have properties such as ease of extraction and protein-substrate interaction that compensate for the deficiency of both class I and class II hydrophobins. Dubey *et al*., (2014) identified three class II hydrophobins from *Clonostachys rosea* strains and gene expression analysis showed a basal expression of all the three genes during growth and development and under nutritional stress conditions. Askolin *et al*., (2001) has previously purified hydrophobin HFBI from the cell wall of *T. reese*i by extraction with 1% SDS, KCl precipitation to remove SDS and hydrophobic interaction chromatography. High pH value was discovered to improve the extraction capacity by possibly increasing the negative charge of the protein. The expression of novel

ANID_05290 and ANID_0737 hydrophobins using the *Pichia* system and its application with β-glucosidases could provide the potential of the hydrophobins to be used as novel agent of lignocellulose substrate accessibility for β-glucosidases.

Biochemical characterization of the purified AN2227.2 and AN1804.2 β-glucosidases was performed with respect to some parameters (such as pH and temperature) influencing the hydrolytic activity of the enzymes (Chapter 4). The *A. nidulans* recombinant β-glucosidases (AN2227.2 and AN1804.2) expressed from *P. pastoris* were isolated, purified and characterized with AN1804.2 in particular having a remarkably broad pH and temperature profile. AN2227.2 and AN1804.2 β-glucosidases were 48 kDa and 100 kDa in size and were most active at pH 6.0 and 5.5 with optimum temperature of 40 and 50 ^{o}C respectively. AN2227.2 β-glucosidase retained 46 % of its residual activity at 30 ^{o}C while AN1804.2 β-glucosidase retained 94 % of its residual activity at 50 ^{o}C which indicated its suitability for cellulose hydrolysis. The kinetic study shows β-glucosidases from both clones to have high affinity for pNPG substrate indicating an interesting characteristic for application in the hydrolysis of lignocellulosic biomass for biofuel production. To my knowledge, in only one other study (Bauer *et al*., 2006) have workers described a partial characterization of β-glucosidase from AN1804.2 clone; this is the first time the purification and characterization of β-glucosidase AN2227.2 is described.

An *Aspergillus niger* strain was isolated from the soil in Nigeria and two encoding sequences for a β-glucosidase gene were amplified from the genomic DNA. The matching DNA sequence of *Aspergillus niger* ATCC 1015 β-glucosidase is under accession number EHA19734.1. There are several reports on the molecular cloning and expression of β-glucosidase genes from *Aspergillus niger* (Meko'o *et al*., 2010; Kamarudin *et al*., 2015) and *Trichoderma reesei* (Sarah *et al*., 2007; Dashtban and Qin, 2012). Meko'o *et al*., (2010) synthesized a 2526 bp gene encoding *Aspergillus niger* β-glucosidase for its

heterologous expression in *Pichia pastoris* using methanol as an inducer. They reported the enzyme to be a monomer with an apparent weight of 90 kDa. Dashtban and Qin (2012) also successfully engineered a thermostable β-glucosidase gene from the fungus *Periconia sp*. into the genome of *T. reesei* QM9414 strain. The engineered *T. reesei* strain showed higher β-glucosidase activity compared to the parent strain and was thermotolerant at temperatures as high as 60 ^{o}C after two-hour incubation.

7.5 Conclusion

A key issue addressed in the present study is that the *Aspergillus* genome holds candidate β-glucosidases that are horizontally transferred from bacteria. Several candidate enzymes such as NFIA_027390, AFL2G_06408, AO090701000841, NFIA_098520 and AO090005000337 were identified as potential β-glucosidases with important properties for cellulose degradation. The plate assay provided a rapid system to detect cellulase enzyme activity in order to screen mainly samples from time course fermentation. Preliminary screening of fungal and bacterial strains identified *B. subtilis, A. niger* F320 and *A. niger* F321 as good and most efficient cellulase/β-glucosidase producing organisms by their ability to degrade CMC. The molecular cloning of ANID_05290.1 and ANID_07327 gene sequences was successful and confirmed the presence of hydrophobin belonging as an intermediary class of hydrophobin pointing to the role these enzymes could play in lignocellulose hydrolysis.

β-glucosidase from AN1804.2 *A. nidulans* clone was revealed in this study as a good source of β-glucosidase for cellulose hydrolysis. The enzyme was expressed in high volumes and has properties that are useful in cellulose hydrolysis. The study also provided evidence to the fact that thermo-tolerance and very broad pH profile of AN1804.2 β-glucosidase could be the reason why the fungus has acquired and kept the bacterial gene.

Part of the thesis also deals with the production and hydrolysis of bacterial cellulose. The study successfully demonstrated the feasibility of using commercial *Aspergillus* cellulase enzyme in the hydrolysis of BC products and this suggests an attractive prospect for the production and recycling of biopolymers from bacterial sources.

In conclusion, the development of an enhanced technology for lignocellulose degradation based on co-production of β-glucosidase (along with endoglucanase and exoglucanase) and hydrophobin may improve access of cellulolytic enzyme to lignocellulose biomass for biofuel production.

7.6 Recommendations for further work

The study in this thesis has highlighted a number of topics on which future studies would be beneficial.

1. Expression and characterization of hydrophobins from ANID_05290.1 and ANID_07327. Further study could involve structure elucidation and property analysis of the unusual ANID_05290.1 and ANID_07327 hydrophobins.
2. Expression and characterization of synthetic NFIA_098520 and Afu6g12010 β-glucosidases.
3. β-glucosidases from the Nigerian isolate of *A. niger* should be expressed, purified, characterized and tested for their activity on plate assay and on pNPG substrate. Because the *A. niger* strain is isolated from a high temperature region of Nigeria, it may possess thermostable qualities which are useful for the bioconversion of lignocellulosic residues at elevated temperatures (Dashtban *et al*., 2009).
4. The production of a cocktail of enzymes made up of engineered β-glucosidase, endoglucanase, exoglucanase and other proteins such as hydrophobin, to allow access to lignocellulose which is hydrolysed to fermentable sugars by the catalytic enzymes, should be attempted. The rapid assay developed here could be used to

quickly test the activity of different cocktails on a variety of substrates. This will help to overcome the recalcitrance of cellulosic biomass conversion.

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Appendices

Appendix 1: Chemicals

Congo red, Carboxymethylcellulose (CMC) of high viscosity type, Trizma base, Cellobiose, Magnesium sulphate ($MgSO_4$), Sodium Chloride (NaCl), Sodium hydroxide (NaOH), Potassium chloride (KCL), Sodium nitrate ($NaNO_3$), Biotin, p-Nitrophenol (pNP), p-Nitrophenol β-D glucopyranoside (pNPG), Biotin, EcoR I from *E. coli*, Phosphatase, Bovine Serum Albumin (BSA), EDTA, Zymolase, DL-Dithiothreito, Sodium dodecyl sulphate (SDS), Sodium citrate ($Na_3C_6H_5O_7$), Potassium acetate (CH_3CO_2K), D-Sorbitol ($C_6H_{14}O_6$), Ammonium acetate ($C_2H_3O_2NH_4$), Chloroform:Isoamyl alcohol 24:1, Ammonium sulphate ($(NH_4)_2SO_4$), DEAE-Sephadex A-50 and Bradford Reagent were all obtained from Sigma-Aldrich United Kingdom. Malt Extract Agar (MEA), Tryptone Soya Agar (TSA), Peptone, Yeast extract, Agar No. 2 and Ringers tablet were supplied by LAB M Limited Lancashire, UK. Dipotassium hydrogen phosphate (K_2HPO_4), Potassium phosphate (K_3PO_4), Disodium phosphate (Na_2HPO_4) and Sodium di- hydrogen phosphate (NaH_2PO_4) were purchased from Acros organics, UK. Glucose was obtained from British Drug House (BDH) UK. Methanol, Citric acid ($C_6H_8O_7$), Acetic acid ($C_2H_4O_2$), Chloroform and Ethanol were all supplied by Fisher Scientific, Loughborough UK. Propanol was from Rathburn chemicals Limited, Scotland. Agarose, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were from Melford Bio-Laboratories Chelsworth, Ipswich UK. The entire reagents used in this study were of analytical grade.

Appendix 2: Stock Solutions/ Media

a. 10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulphate without amino acids)

Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulphate and without amino acids in 1000 ml of water and filter sterilize. Heat the solution to dissolve YNB completely in water. Store at 4°C. Alternatively, use 34 g of YNB without ammonium sulphate and amino acids and 100 g of ammonium sulphate. The shelf life of this solution is approximately one year. If you are using the YNB pouch included in the kit, follow the directions on the pouch.

Note: *Pichia* cells exhibit optimal growth with higher YNB concentrations; therefore, the amount of YNB used in this kit is twice as concentrated as YNB formulations for *Saccharomyces.*

b. 500X B (0.02% Biotin)

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at 4°C. The shelf life of this solution is approximately one year.

c. 100X H (0.4% Histidine)

Dissolve 400 mg of L-histidine in 100 ml of water. Heat the solution, if necessary, to no greater than 50°C in order to dissolve. Filter sterilizes and store at 4°C. The shelf life of this solution is approximately one year.

d. 10X D (20% Dextrose)

Dissolve 200 g of D-glucose in 1000 ml of water. Autoclaves for 15 minutes or filter sterilize. The shelf life of this solution is approximately one year.

e. 10X M (5% Methanol)

Mix 5 ml of methanol with 95 ml of water. Filter sterilizes and store at 4°C. The shelf life of this solution is approximately two months.

f. 10X GY (10% Glycerol)

Mix 100 ml of glycerol with 900 ml of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is greater than one year.

g. 1 M potassium phosphate buffer, pH 6.0:

Combine 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 and confirm that the pH

= 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Sterilize by autoclaving and store at room temperature. The shelf life of this solution is greater than one year.

h. **YPD or YEPD Yeast Extract Peptone Dextrose Medium** (1 litre)

1% yeast extract

2% peptone

2% dextrose (glucose)

Note: If you are using the YP Base Medium or the YP Base Agar medium pouches included with the Original Pichia Expression Kit, follow the directions on the pouch.

1. Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water.

2. Autoclave for 20 minutes on liquid cycle.

3. Add 100 ml of 10X D.

The liquid medium is stored at room temperature. Store YPD slants or plates are at 4°C.

The shelf life is several months.

i. BMGH and BMMH Buffered Minimal Glycerol
Buffered Minimal Methanol (1 liter)

100 mM potassium phosphate, pH 6.0

1.34% YNB

4 × 10–5% biotin

1% glycerol or 0.5% methanol

1. Autoclave 690 ml water for 20 minutes on liquid cycle.

2. Cool to room temperature, then add the following and mix well:

100 ml 1 M potassium phosphate buffer, pH 6.0

100 ml 10X YNB

2 ml 500X B

100 ml 10X GY

3. For BMMH, add 100 ml 10X M instead of glycerol.
4. To add histidine, add 10 ml of 100 X H stock solutions. Mix and store at 4°C.
5. Store media at 4°C. The shelf life of this solution is approximately two months.

ii. BMGY and BMMY Buffered Glycerol-complex Medium
Buffered Methanol-complex Medium (1 liter)

1% yeast extract

2% peptone

100 mM potassium phosphate, pH 6.0

1.34% YNB

4 × 10-5% biotin

1% glycerol or 0.5% methanol

1. Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water.
2. Autoclave 20 minutes on liquid cycle.
3. Cool to room temperature, then add the following and mix well:

100 ml 1 M potassium phosphate buffer, pH 6.0

100 ml 10X YNB

2 ml 500X B

100 ml 10X GY

4. For BMMY, add 100 ml 10X M instead of glycerol.
5. Store media at 4°C. The shelf life of this solution is approximately two months.

iii. Luria Bertani Media: The LB medium was prepared according to Table iv below and was sterilized at 121 ^{o}C for 20 minutes. Before the addition of antibiotics to the sterilized medium, it was cooled down to room temperature with either 100 µg/ml of ampicillin or 25 µg/ml of

zeocin under sterile conditions. Agar plates were prepared by adding 15 g/l agar to the above media before autoclaving. Agar plates containing antibiotics were usually stored at 4 ^{o}C.

iv. Composition of LB and low salt LB medium

Component	Concentration (g/l)
Yeast extract	5
Peptone (Tryptone)	10
NaCl	10 (5 for low salt LB medium)

v. Minimal medium

Materials (per litre preparation)

i.	$NaNO_3$	1g
ii.	K_2HPO_4	1g
iii.	KCL	1g
iv.	$MgSO_4$	0.5g
v.	Agar No 2	15g

Materials (As may be required)

i.	Glucose	1%w/v
ii.	Cellobiose	5g
iii.	Cellulose	5g
iv.	Ether	1ml (for sterilization of carbon source)

Sterilization of carbon source

i. To a sterile flask, add 5g of carbon source
ii. Add 1ml of ether for 10 – 15 minutes
iii. Loose cotton wool and leave to evaporate in fume hood
iv. Add autoclaved agar.

Vitamin Solution

i.	P-amino benzoic acid	80mg
ii.	Inositol	80mg
iii.	Nicotinic acid	20mg
iv.	Ca-D-panthothenate	120mg (panthetonic acid, Ca-salt)
v.	Pyridoxine	50mg
vi.	Riboflavin	20mg
vii.	Choline chloride	280mg
viii.	Putrescine	400mg
ix.	Biotin stock	2ml
x.	Distilled water	198ml

Casein Hydrolysate

i.	Difco Casamino acids	37.5g
ii.	Distilled water	250ml

Appendix 3: Primer design

i. On NCBI web page, click on BLAST.

ii. Click on microbes.

iii. Click on blastp (or blastn) and paste your FASTA sequence.

iv. For organism, type specie name e.g. *B. subtilis*.

v. Click on Accession No. of best match.

vi. Under "CDS" (coding sequence - just before the sequence), click on Gene ID. Alternatively under "Related information" (to the right hand side), click on "Gene".

vii. Right click on the gene (i.e. the red bar), click on primer search tool.

viii. On the "Primer-BLAST" page, include some bases on the "Range" (for instance, 300 bases on both sides).

ix. Click on "Get primers" option to generate your primers.

Appendix 4: Buffers preparations

1. Citrate Buffer
   A. 0.1 M solution of citric acid (21.01 g in 1 L)
   B. 0.1 M solution of sodium citrate (29.41 g $C_6H_5O_7Na_3.2H_2O$ in 1 L)

   x mL of A + y mL of B, diluted to a total of 100 mL

X	Y	pH
46.5	3.5	3.0
33.0	17.0	4.0
25.5	24.5	4.5
20.5	29.5	5.0
13.7	36.3	5.5
9.5	41.5	6.0

2. Phosphate Buffer
   A. 0.2 M solution of monobasic sodium phosphate (27.8 g in 1 L)
   B. 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1 L)

   x mL of A + y mL of B, diluted to a total of 200 mL

X	Y	pH
68.5	31.5	6.5
39.0	61.0	7.0
5.3	94.7	8.0

3. Glycine-NaOH Buffer
   A. 0.2 M solution of glycine (15.01 g in 1 L)
   B. 0.2 M NaOH

   50 mL of A + x mL of B, diluted to a total of 200 mL

X	pH
8.8	9.0
32.0	10.0

Appendix 5: Some terms and calculations in purification of β-glucosidase

Activity is defined as the amount of β-glucosidase enzyme needed to liberate 1µM of p-Nitrophenol (pNP) per minute under the standard assay conditions.

1. **Specific activity** = Unit of the enzyme/Total mg of protein
2. **Total activity** = Specific activity X Total mg protein in fraction
3. **Purification fold** = Specific activity of a given fraction/Original specific activity
4. **Recovery or yield (%)** = Total activity of a given fraction/Total activity of the original mixture X 100

Appendix 6: Nucleotide sequence of read across cloning site for NFIA_027390 clone in pPICZ A vector

>NFIA_027390A

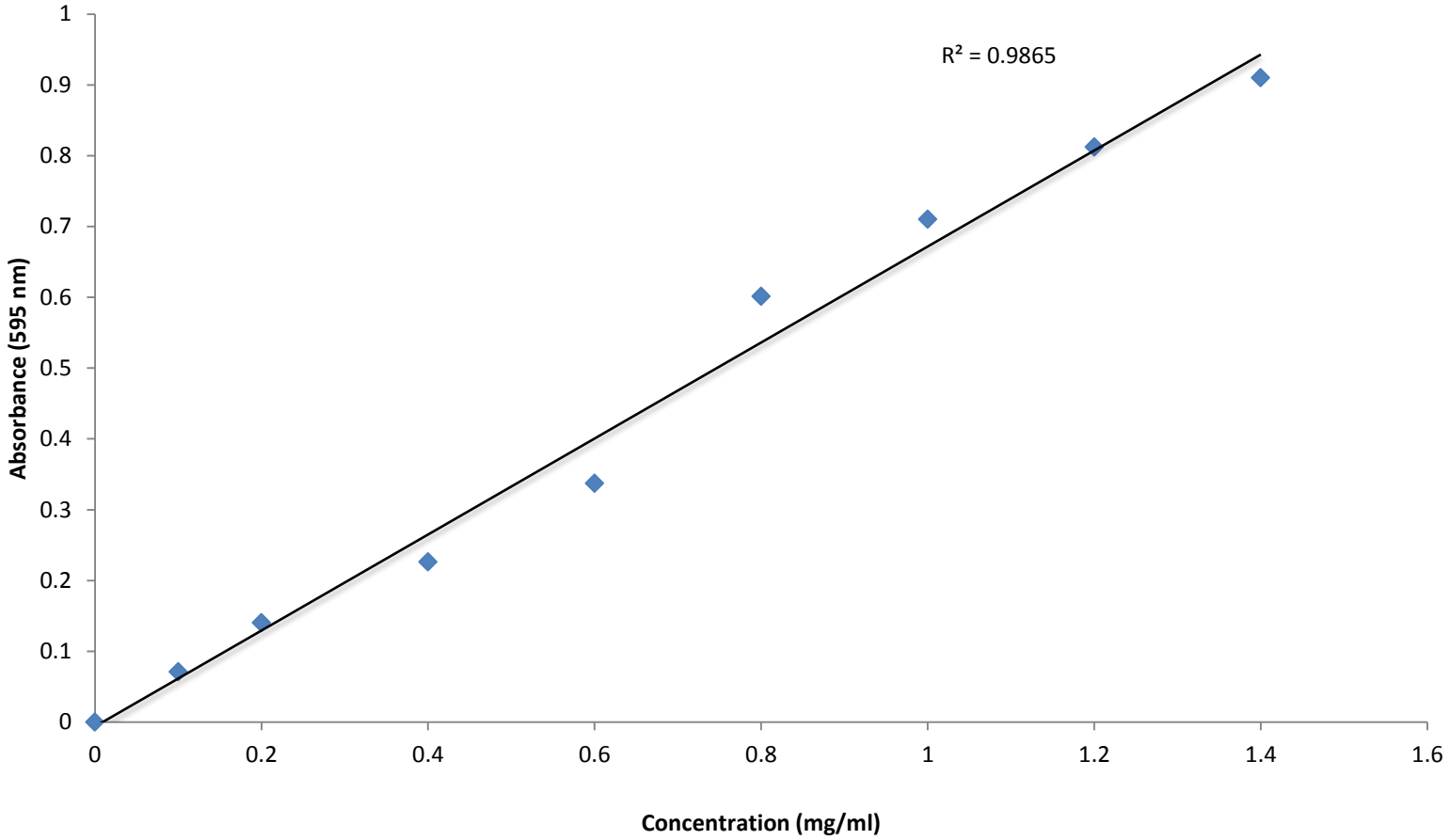
ACACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCACGTGGCCCACCNGC
TCGGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCTTGGGCCCGAACAAAAACTCATCTCAGA
AGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGAGTTTTAGCCTTAGACATG
ACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAG
GATGTCAGAATGCCATTTGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACC
TATATAGTATAGGATTTTTTTTGTCATTTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTA
TCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTTCT
TGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGTGCGGATCCCC
CACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCG
CATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATT

Appendix 7: Nucleotide sequence of read across cloning site for NFIA_027390 clone in pPICZ B vector

>NFIA_027390B
CGACACTTGAGAGAAAACAACTAATTATTCGAAACGAGAATTCACGTGGCCCAGCCGGCCGTC
TCGGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGA
TGATCTGAATAGCGCCGTCCACCATCATCATCATCATTGAGTTTGTAGCCTTAAACATGGCT
GTTCCTCACTTCAAGTTGGGCACTTACAAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGAT
GTCAGAATGCCTTTTGCCTGAAAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTAT
ATAGTATAGGATTTTTTTTGTCATTTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCT
CGCAGCTGATGAATATGTTGTGGGAGGGGTTTGGGAAAATCATTCTAGTTTGATGTTTTTCTTGG
TATTTCCCACTCCTCTTCATAGTACAGAATATTAGATGAGACCTTCGGTTGTGCGGATCCCCCAC
ACACCATATCTTCAAGATGTTTCTACTCCTTTTTTACTCTTCAAGATTTTCTCGGACTCCGCGCAT
CGCCGTACCACTTCAAAACACCCAAGCACAGCATACTATATTTCCCCTCTTTCTTCCTCTATGGT
GTCGTTAATTACCCGTACTAGAGGTTTGGAAAAAAAGAGACCGCCTCGCTTCTTTTTCTTCGTCA
AAGAGGCAATAAAATTTTTATCACGCTTCTTCTTCTGAAAATTTTTTTTTTTGATTTTTTTCTCTTT
CGATGACCTCCCATTGATATTTATTAATAAACGGTCTTTTCNCAAGTTTCATTCATTTTTGTGTAC
TATTACACTTTTTTACTCTGCTCATTAAGAAAGCATACATCTATAGGCGGTGTTGACAATTAATC
TCGGTAGTATCGGCT

Appendix 8: BSA standard curve

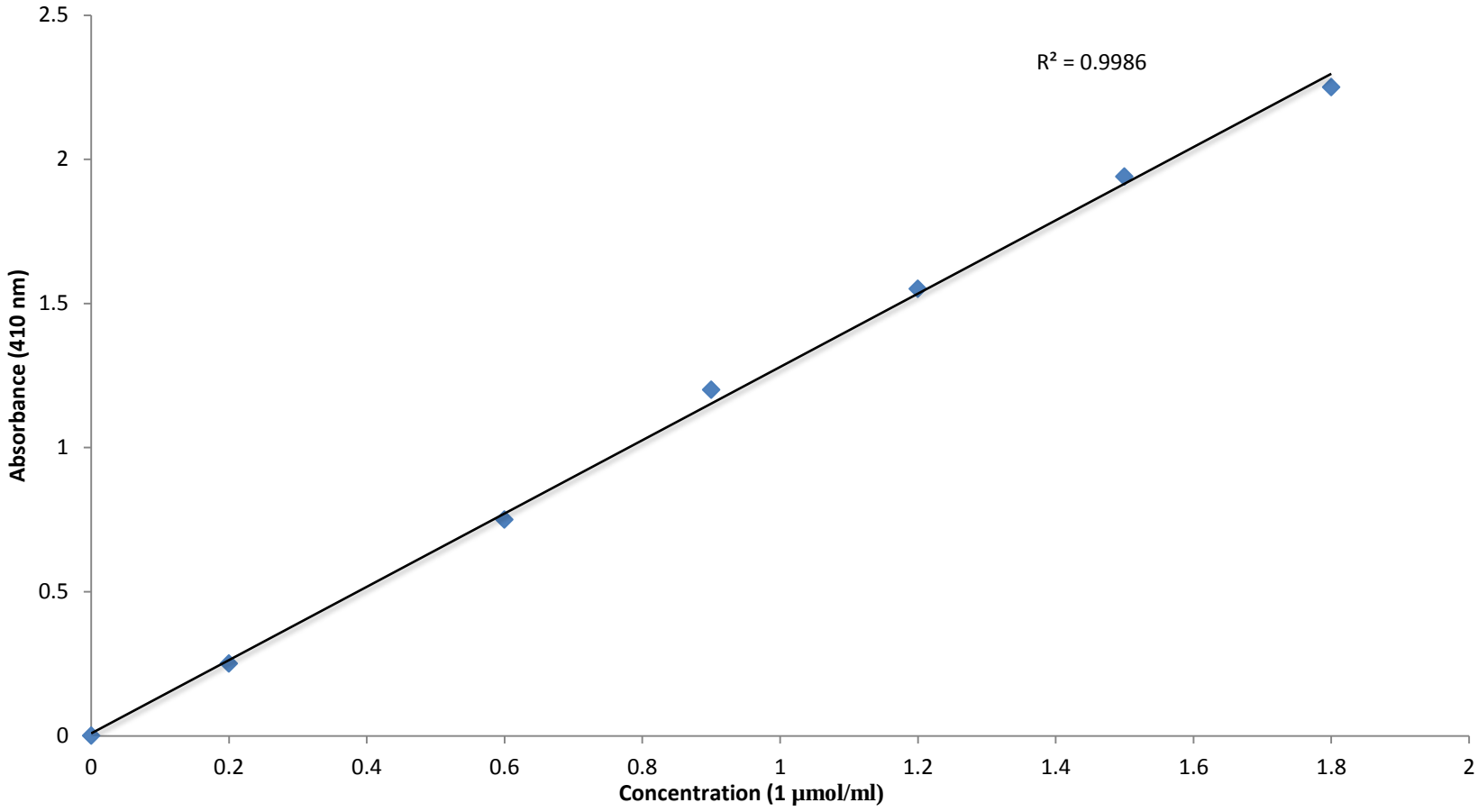
Tube No	Final BSA concentration (mg/ml)	Amount of standard BSA needed (µL)	Amount of dH_2O needed (µL)	Absorbance (595 nm)
1	0.0	0	100	0.00
2	0.1	5	95	0.071
3	0.2	10	90	0.140
4	0.4	20	80	0.226
5	0.6	30	70	0.337
6	0.8	40	60	0.601
7	1.0	50	50	0.710
8	1.2	60	40	0.812
9	1.4	70	30	0.910



BSA standard curve

Appendix 9: p-Nitrophenol standard curve

Tube No	pNP concentration (1 µmol/ml)	NaOH (ml)	dH_2O (ml)	Absorbance (410 nm)
1	0.0	1.8	1.8	0.0
2	0.2	1.6	1.8	0.25
3	0.6	1.2	1.8	0.75
4	0.9	0.9	1.8	1.20
5	1.2	0.6	1.8	1.55
6	1.5	0.3	1.8	1.94
7	1.8	0.0	1.8	2.25



p-Nitrophenol standard curve

Appendix 10: Time course for β-glucosidase Production by *A. nidulans* AN2227 and AN1804

Serial No.	Time	AN2227 Protein conc. (mg/ml)	AN1804 Protein conc. (mg/ml)
1	0	0.00 ± 0.0	0.00 ± 0.0
2	6	0.03 ± 0.0	0.08 ± 0.01
3	12	0.33 ± 0.01	0.13 ± 0.02
4	24	0.37 ± 0.13	0.30 ± 0.10
5	48	0.67 ± 0.03	0.56 ± 0.11
6	72	0.22 ± 0.05	0.42 ± 0.16

Appendix 11: Time course for β-glucosidase activity by *A. nidulans* AN2227 and AN1804

Serial No.	Time	AN2227 Activity (µmoles/ml/min)	AN1804 Activity (µmoles/ml/min)
1	0	0.00 ± 0.0	0.00 ± 0.0
2	6	0.13 ± 0.04	0.57 ± 0.03
3	12	0.17 ± 0.05	1.45 ± 0.02
4	24	0.39 ± 0.06	1.59 ± 0.03
5	48	0.52 ± 0.03	1.78 ± 0.06
6	72	0.45 ± 0.03	1.61 ± 0.15

Appendix 12: Anion-exchange chromatography for AN2227 β-glucosidase on DEAE-Sephadex A-50

Fraction No	Total protein Abs (595 nm)	Total protein (mg/ml)	Enzyme activity absorbance (410 nm)	Enzyme activity (µm/ml/min)	Specific activity (µm/ml/min/mg protein)
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	0.06	0.10	0.04	0.03	0.30
8	0.06	0.10	0.10	0.08	0.80
9	0.11	0.15	0.20	0.16	1.07
10	0.12	0.16	0.23	0.18	1.13
11	0.22	0.31	0.47	0.37	1.19
12	0.20	0.30	0.68	0.53	1.77
13	0.15	0.20	0.41	0.31	1.55
14	0.09	0.13	0.23	0.18	1.38
15	0.07	0.11	0.20	0.16	1.45
16	0.07	0.11	0.18	0.14	1.27
17	0.10	0.14	0.17	0.13	0.93
18	0.07	0.11	0.18	0.14	1.27
19	0.11	0.15	0.17	0.13	0.87
20	0.10	0.14	0.12	0.10	0.71
21	0.09	0.13	0.07	0.05	0.39
22	0.06	0.10	0.02	0.01	0.10
23	-	-	-	-	-

Appendix 13: Anion-exchange chromatography for AN1804 β-glucosidase on DEAE-Sephadex A-50

Fraction No	Total protein Abs (595 nm)	Total protein (mg/ml)	Enzyme activity absorbance (410 nm)	Enzyme activity (µm/ml/min)	Specific activity (µm/ml/min/mg protein)
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	-	-	-	-	-
9	-	-	-	-	-
10	0.07	0.11	1.30	0.24	2.18
11	0.12	0.17	3.89	1.56	9.18
12	0.61	0.88	4.01	1.58	1.80
13	0.26	0.38	3.30	1.00	2.63
14	0.26	0.38	1.77	0.61	1.61
15	0.24	0.34	3.07	0.52	1.53
16	0.24	0.34	3.06	0.50	2.08
17	0.22	0.31	2.13	0.10	0.32
18	0.18	0.26	0.60	0.05	0.19
19	0.08	0.11	0.24	0.02	0.18
20	0.03	0.03	0.06	0.01	0.33
21	0.03	0.03	0.06	0.01	0.33
22	0.03	0.03	0.01	0.00	0.00
23	-	-	-	-	-

Appendix 14: Effect of pH on β-glucosidase activity

Serial No	pH	AN2227 Activity (µmoles/ml/min)	AN1804 Activity (µmoles/ml/min)
1	3	0.43 ± 0.02	1.50 ± 0.13
2	4	0.46 ± 0.01	1.65 ± 0.02
3	4.5	0.53 ± 0.05	1.67 ± 0.03
4	5	0.55 ± 0.02	1.72 ± 0.07
5	5.5	0.59 ± 0.01	1.77 ± 0.08
6	6	0.60 ± 0.01	1.61 ± 0.04
7	6.5	0.59 ± 0.03	1.51 ± 0.15
8	7	0.51 ± 0.00	1.51 ± 0.03
9	7.5	0.53 ± 0.02	1.50 ± 0.03
10	8	0.47 ± 0.00	1.46 ± 0.01
11	9	0.29 ± 0.03	1.35 ± 0.09
12	10	0.21 ± 0.00	1.22 ± 0.04

Appendix 15: Effect of temperature on β-glucosidase activity

Serial No	Temperature (^{o}C)	AN2227 Activity (µmole/ml/min)	AN1804 Activity (µmole/ml/min)
1	10	0.24 ± 0.04	1.22 ± 0.03
2	20	0.21 ± 0.06	1.73 ± 0.02
3	30	0.46 ± 0.07	1.74 ± 0.12
4	40	0.77 ± 0.07	1.80 ± 0.09
5	50	0.76 ± 0.02	1.95 ± 0.06
6	60	0.28 ± 0.04	1.61 ± 0.10
7	70	0.26 ± 0.09	1.31 ± 0.00
8	80	0.20 ± 0.01	0.73 ± 0.05
9	90	0.15 ± 0.04	0.12 ± 0.02

Appendix 16: Thermostability of AN2227 β-glucosidase

Temperature (^oC)	Temperature (^oK)	Activity (µmole/ml/min)	Log V	1/T x 10^{-3}
10	283	0.58 ± 0.06	- 0.237	3.5634
20	293	0.56 ± 0.07	- 0.252	3.413
30	303	0.29 ± 0.06	- 0.538	3.300
40	313	-	-	3.195
50	323	-	-	3.096
60	333	-	-	3.003
70	343	-	-	2.916
80	353	-	-	2.833
90	363	-	-	2.755

Appendix 17: Residual activity of AN2227 β-glucosidase

Temperature (^oC)	Activity (µmole/ml/min)	Residual activity (%)
10	0.58	103.57
20	0.56	100
30	0.26	46.43

Appendix 18: Thermostability of AN1804 β-glucosidase

Temperature (^oC)	Temperature (^oK)	Activity (µmole/ml/min)	Log V	1/T x 10^{-3}
10	283	1.53 ± 0.05	0.149	3.5634
20	293	1.55 ± 0.07	0.190	3.413
30	303	1.50 ± 0.11	0.176	3.300
40	313	1.48 ± 0.05	0.170	3.195
50	323	1.46 ± 0.08	0.164	3.096
60	333	-	-	3.003
70	343	-	-	2.916
80	353	-	-	2.833
90	363	-	-	2.755

Appendix 19: Residual activity of AN1804 β-glucosidase

Temperature (^{o}C)	Activity (µmole/ml/min)	Residual activity (%)
10	1.53	98.71
20	1.55	100
30	1.50	96.77
40	1.48	95.48
50	1.46	94.19

Residual activity = x/y x 100

Y = Reference activity at room temperature

X = Activity at other temperature

Appendix 20: Determination of kinetic constants of *A. nidulans* AN2227 β-glucosidase

Substrate Concentration (mg/ml)	Substrate Concentration (mM) [S]	Enzyme activity (µmole/ml/min)	1/[S]	1/V
10	33.19	0.02 ± 0.01	0.03	50
20	66.39	0.04 ± 0.01	0.015	25
40	132.78	0.06 ± 0.00	0.0080	16.67
60	199.17	0.08 ± 0.01	0.0050	12.5
80	265.95	0.09 ± 0.03	0.0038	11.11
100	331.95	0.12 ± 0.01	0.0030	8.33
120	398.34	0.13 ± 0.02	0.0025	7.69
140	464.73	0.12 ± 0.03	0.0022	8.00

Intercept = 1/Vmax

4.9 = 1/Vmax

Vmax = 1/4.9 = 0.20 µmoles/ml/min

-2.4 = 1/Km

Km = 0.42 mM

To convert mg/ml to mM:

Conc. (M) = (moles X 1000)/volume

Moles = Mass/Molar mass

E.g. Conc. (M) = 10/301.25 X 1000

= 33.19 mM

(301.25 = Molar mass of pNPG)

Appendix 21: Determination of kinetic constants of *A. nidulans* AN1804 β-glucosidase

Substrate Concentration (mg/ml)	Substrate Concentration (mM) [S]	Enzyme activity (µmole/ml/min)	1/[S]	1/V
10	33.19	0.16 ± 0.01	0.03	6.25
20	66.39	0.22 ± 0.05	0.015	4.55
40	132.78	0.51 ± 0.05	0.0080	1.96
60	199.17	0.77 ± 0.05	0.0050	1.30
80	265.95	1.07 ± 0.05	0.0038	0.93
100	331.95	1.22 ± 0.03	0.0030	0.82
120	398.34	1.20 ± 0.11	0.0025	0.83
140	464.73	1.22 ± 0.08	0.0022	0.82

Intercept = 1/Vmax

0.49 = 1/Vmax

Vmax = 1/0.49 = 2.04 µmoles/ml/hour

-1.7 = 1/Km

Km = 0.59 mM

How to calculate kinetic constants (Vmax and Km) using Lineweaver-Burk plot

The Lineweaver-Burk method uses reciprocal values of substrate concentration (1/[S]) and reaction velocity (1/V) to obtain a straight line graph. The graph allows for the calculation of both the Vmax and the Michaelis-Menten constant (Km) through linear regression of the data points.

1. Calculate the inverse of both set of data. Divide by each data point.
2. Use a graphing calculator to plot a graph with the inverse values. The 1/[S] values are plotted along the x-axis and 1/V is plotted along the y-axis.
3. Complete a linear regression for the data points. The linear regression yields the equation for the straight line, Y = MX + C with the intercept at 1/Vmax and slope = Km/Vmax. The intersection of the plotted line with the abscissa (x-axis) gives the value of -1/Km. Vmax is equal to the inverse of the y-intercept value.

Appendix 22: Effect of cation on β-glucosidase activity

Serial No	Cofactor (0.02M)	AN2227 Activity (µmole/ml/min)	AN1804 Activity (µmole/ml/min)
1	Control	0.60 ± 0.012	1.65 ± 0.025
2	$MgCl_2$	0.61 ± 0.006	1.62 ± 0.059
3	$CoCl_2$	0.67 ± 0.012	1.72 ± 0.017
4	$FeCl_3$	0.73 ± 0.006	1.82 ± 0.025
5	$CaCl_2$	0.72 ± 0.010	1.73 ± 0.044
6	$FeCl_2$	0.77 ± 0.012	1.82 ± 0.021
7	$HgCl_2$	0.21 ± 0.064	1.09 ± 0.006
8	$ZnCl_2$	0.67 ± 0.010	1.77 ± 0.047

Appendix 23: Effect of Temperature (^{o}C) on fungal growth (mm)

Strain/T (^{o}C)	25	30	35	40	45	50
A. niger F.7	3.73 ± 0.1	4.70 ± 0.1	4.70 ± 0.2	4.73 ± 0.4	1.30 ± 0.1	0.00
A. niger F.9	3.37 ± 0.2	3.83 ± 0.1	4.27 ± 0.1	3.60 ± 0.1	2.07 ± 0.1	0.00
A. niger F.10	2.47 ± 0.1	3.63 ± 0.2	0.67 ± 0.1	0.00	0.00	0.00
D. arenaria F200	5.63 ± 0.1	7.33 ±0.2	7.33 ± 0.2	1.30 ± 0.3	0.00	0.00
D. salina F201	3.20 ± 0.1	2.60 ± 0.1	0.00	0.00	0.00	0.00
D. salina F202	3.93 ±0.2	4.63 ± 0.4	6.47 ± 0.1	5.37 ± 0.2	4.77 ± 0.1	0.00
D. salina F203	3.73 ± 0.1	3.50 ± 0.1	1.07 ± 0.1	0.00	0.00	0.00
D. salina F204	4.77 ± 0.3	4.10 ± 0.1	0.00	0.00	0.00	0.00
D. arenaria F205	3.37 ± 0.1	3.10 ± 0.3	1.30 ± 0.3	0.00	0.00	0.00
D. arenaria F206	4.83 ± 0.1	4.47 ± 0.2	1.07 ± 0.1	0.00	0.00	0.00
D. arenaria F207	4.97 ± 0.2	6.77 ± 0.1	5.27 ± 0.2	0.00	0.00	0.00
D. arenaria F208	4.83 ± 0.1	5.87 ± 0.3	3.73 ± 0.3	2.10 ± 0.4	0.00	0.00
D. salina F210	2.57 ± 0.1	2.47 ± 0.1	0.97 ± 0.1	0.00	0.00	0.00
A. niger F212	3.67 ± 0.2	5.17 ± 0.2	6.73 ± 0.3	5.27 ± 0.3	4.80 ± 0.1	0.00
A. niger F287	4.03 ± 0.1	5.60 ± 0.4	6.20 ± 0.2	3.60 ± 0.2	0.90 ± 0.1	0.00
A. niger F320	5.07 ± 0.6	6.50 ± 0.2	6.33 ± 0.2	3.37 ± 0.2	1.57 ± 0.5	0.00
A. niger F321	5.10 ± 0.3	7.57 ± 0.2	7.93 ± 0.1	5.40 ± 0.3	3.47 ± 0.1	0.00
*A. nidulans*L19	4.00 ± 0.2	5.33 ± 0.3	7.37 ± 0.2	7.07 ± 0.1	4.87 ± 0.1	0.00
*A. nidulans*L20	3.13 ± 0.1	0.00	0.00	0.00	0.00	0.00
*A. nidulans*POL1	3.17 ± 0.1	3.97 ± 0.1	4.33 ± 0.2	4.83 ± 0.2	3.57 ± 0.3	0.00
*A. nidulans*GO281	4.23 ± 0.1	5.53 ± 0.1	6.93 ± 0.1	5.83 ± 0.2	4.80 ± 0.3	0.00

Results are mean values of three replicates. ± = Standard deviation

Appendix 24: FGSC Strains zone of hydrolysis (mm) – YEPD

Strain/Time (hr)	0	3	6	9	12	15	18	24	48	72	96
AN0712	-	-	-	-	9.56 ± 0.51	10.78 ± 0.39	11.44 ± 0.77	12.67 ± 0.88	14.56 ± 1.39	14.89 ± 0.84	15.11 ± 1.17
AN1551	-	-	-	-	9.89 ± 0.51	10.55 ± 0.69	11.00 ± 1.00	12.78 ± 1.02	14.55 ± 1.95	15.89 ± 2.84	14.11 ± 1.26
AN1804	-	-	-	-	9.11 ± 0.84	10.66 ± 0.58	11.22 ± 0.39	12.67 ± 0.88	14.33 ± 0.88	15.89 ± 1.95	14.22 ±1.07
AN2227	-	-	-	-	9.78 ± 1.15	10.22 ± 1.02	10.44 ± 1.50	13.56 ± 1.39	14.89 ± 1.17	16.00 ± 2.18	13.78 ± 1.26
AN2612	-	-	-	-	10.56 ± 0.51	10.67 ± 0.58	11.22 ± 0.51	13.56 ± 1.17	14.67 ± 1.21	16.11 ± 1.90	14.56 ± 0.94
P. pastoris 323	-	-	-	-	8.33 ± 1.20	8.67 ± 1.53	9.67 ± 1.85	11.11 ± 1.07	11.45 1.07	13.56 ± 1.83	12.67 ± 0.88

Note: Results are mean of means in triplicate ± standard deviation.

Appendix 25: FGSC Strains Viable Count, CFU – YEPD

Strain /Time (hr)	0	3	6	9	12	15	18	24	48	72	96
AN0712	6.74 ± 0.13	6.68 ± 0.06	6.85 ± 0.07	6.79 ± 0.06	7.45 ± 0.23	7.54 ± 0.23	7.81 ± 0.12	8.30 ± 0.35	8.58 ± 0.20	8.62 ± 0.19	8.51 ± 0.10
AN1551	6.57 ± 0.12	6.59 ± 0.24	6.55 ± 0.32	6.67 ± 0.21	7.59 ± 0.14	7.67 ± 0.04	7.70 ± 0.22	8.13 ± 0.28	8.49 ± 0.02	8.57 ± 0.09	8.65 ± 0.06
AN1804	6.60 ± 0.23	6.64 ± 0.28	6.56 ± 0.16	5.99 ± 1.17	7.59 ± 0.12	7.69 ± 0.11	7.91 ± 0.07	8.20 ± 0.09	8.52 ± 0.13	8.66 ± 0.05	8.67 ± 0.11
AN2227	6.66 ± 0.11	6.77 ± 0.04	6.71 ± 0.10	6.81 ± 0.03	7.50 ± 0.16	7.67 ± 0.10	7.87 ± 0.11	8.45 ± 0.14	8.48 ±0.08	8.52 ±0.24	8.55 ± 0.10
AN2612	6.54 ± 0.34	6.59 ± 0.15	6.57 ± 0.30	6.75 ± 0.10	7.41 ± 0.21	7.69 ± 0.08	7.92 ± 0.10	8.15 ± 0.07	8.39 ± 0.13	8.51 ± 0.17	8.76 ± 0.19
P. pastoris 323	6.78 ± 0.17	6.86 ± 0.07	6.96 ± 0.10	6.90 ± 0.16	7.93 ± 0.14	8.30 ± 0.27	8.62 ±0.03	8.80 ± 0.04	8.96 ± 0.07	9.03 ± 0.08	9.02 ±0.19

Note: Results are mean of means in triplicate ± standard deviation.

Appendix 26: FGSC Strains zone of hydrolysis (mm) – BMMY

Strain /Time (hr)	0	3	6	9	12	15	18	24	48	72	96
AN0712	-	-	-	-	-	-	-	9.11 ± 0.84	11.11 ± 1.02	11.11 ± 1.39	10.56 ± 0.77
AN1551	-	-	-	-	-	-	-	9.33 ± 1.20	10.78 ± 0.69	11.22 ± 1.02	10.89 ± 0.70
AN1804	-	-	-	-	-	-	-	9.78 ± 0.39	11.33 ± 0.34	11.56 ± 1.71	11.11 ±1.50
AN2227	-	-	-	-	-	-	-	9.56 ± 0.51	10.78 ± 0.69	11.44 ± 1.02	11.22 ± 0.84
AN2612	-	-	-	-	-	-	-	9.22 ± 1.07	10.66 ± 0.56	11.22 ± 1.35	11.11 ± 1.17
P. pastoris 323	-	-	-	-	-	-	-	-	-	-	-

Note: Results are mean of means in triplicate ± standard deviation.

Appendix 27: FGSC Strains Viable Count, CFU – BMMY

Strain/ Time (hr)	0	3	6	9	12	15	18	24	48	72	96
AN0712	6.63 ± 0.19	6.64 ± 0.23	6.60 ± 0.21	6.61 ± 0.16	7.45 ± 0.23	7.49 ± 0.20	7.77 ± 0.25	8.34 ± 0.17	8.35 ± 0.11	8.45 ± 0.10	8.59 ± 0.06
AN1551	6.77 ± 0.19	6.68 ± 0.18	6.76 ± 0.14	6.83 ± 0.19	7.26 ± 0.04	7.37 ± 0.10	7.47 ± 0.10	8.06 ± 0.24	8.07 ± 0.26	7.83 ± 0.59	8.18 ± 0.71
AN1804	6.74 ± 0.23	6.75 ± 0.30	6.76 ± 0.26	6.86 ± 0.23	7.37 ± 0.02	7.47 ± 0.33	7.80 ± 0.14	8.38 ± 0.18	8.49 ± 0.08	8.52 ± 0.14	8.60 ± 0.26
AN2227	6.68 ± 0.16	6.58 ± 0.16	6.68 ± 0.21	6.78 ± 0.09	7.16 ± 0.30	7.36 ± 0.10	7.69 ± 0.18	8.22 ± 0.30	8.78 ± 0.80	8.84 ± 0.86	8.79 ± 0.78
AN2612	6.86 ± 0.16	6.75 ± 0.27	6.88 ± 0.24	6.90 ± 0.10	7.33 ± 0.26	7.57 ± 0.16	7.66 ± 0.13	8.42 ± 0.16	8.54 ± 0.22	8.62 ± 0.07	8.74 ± 0.07
P. pastoris 323	6.95 ± 0.13	7.04 ± 0.10	6.98 ± 0.03	7.07 ± 0.11	7.73 ± 0.13	7.84 ± 0.15	7.88 ± 0.02	8.09 ± 0.16	8.36 ± 0.15	8.54 ± 0.08	8.70 ± 0.06

Note: Results are mean of means in triplicate ± standard deviation.

Appendix 28

Summary of *Aspergillus* β-glucosidase classified according to GH family, amino acid length, number of introns and signal peptide cleavage position

Accession No.	GH family	Amino acid length (Bases)	Total No of Introns	Signal peptide Cleavage site (Position between)
Anid_10375	1	486	1	Absent
Anid_10124	1	483	4	26 – 27
Anid_02217	3	779	9	24 - 25
Anid_04102	3	853	6	19 - 20
Anid_00712	3	845	3	Absent
Anid_02828	3	737	4	19 - 20
Anid_02612	3	838	3	Absent
Anid_06652	3	1023	2	Absent
Anid_01804	3	618	0	19 - 20
Anid_07865	3	805	4	Absent
Anid_09183	1	605	2	15 - 16
Anid_02227	3	838	2	Absent
Anid_10482	3	868	2	20 - 21
Anid_05976	3	819	1	20 - 21
Anid_03903	3	725	1	Absent
Anid_07396	3	772	6	21 - 22
Anid_8404	3	763	0	23 - 24
Anid_02359	3	800	0	20 - 21
Anid_00479	3	517	5	Absent
Anid_03949	3	723	4	21 - 22
Anid_07915	3	799	2	Absent
Anid_01416	3	923	3	Absent
Anid_02599	3	385	4	Absent

Afu5g07190	3	838	2	Absent
Afu5g07080	3	797	9	24 - 25
Afu6g08700	3	888	1	19 - 20
Afu6g03570	3	1033	3	Absent
Afu3g00230	3	833	6	Absent
Afu1g05770	3	873	8	19 - 20
Afu6g14490	3	829	2	Absent
Afu8g02100	3	806	1	20 - 21
Afu1g17410	3	769	6	22 - 23
Afu7g06140	3	739	2	17 - 18
Afu6g11910	3	856	3	Absent
Afu7g00240	3	767	4	Absent
Afu8g06970	1	583	0	19 - 20
Afu6g14600	1	503	0	Absent
Afu1g14710	1	483	4	Absent
Afu1g16400	1	497	2	Absent
Afu3g12600	1	488	1	Absent
Afu6g12010	3	409	0	Absent
Afu3g02090	3	771	0	25 -26
Afu1g16920	3	792	0	20 - 21
ATEG_02657	1	570	2	17 - 18
ATEG_03047	3	861	6	19 - 20
ATEG_07931	3	736	4	21 - 22
ATEG_10320	3	782	5	19 - 20
ATEG_02806	3	757	1	Absent
ATEG_10274	3	790	4	20 - 21
ATEG_07121	3	1466	7	Absent

ATEG_07419	3	729	3	17 - 18
ATEG_00687	1	481	4	Absent
ATEG_06617	3	867	2	19 - 20
ATEG_09329	3	839	2	Absent
ATEG_04135	1	487	1	Absent
ATEG_09314	3	795	9	24 - 25
ATEG_02713	3	817	1	20 - 21
ATEG_04069	3	1421	6	Absent
ATEG_00157	3	860	3	Absent
ATEG_02724	3	762	0	Absent
ATEG_08027	3	794	8	17 – 18
ATEG_09052	3	765	0	25 - 26
ATEG_07383	3	908	2	26 - 27
ATEG_05106	3	776	1	Absent
AFL2G_09187	3	866	2	Absent
AFL2G_10322	3	874	5	Absent
AFL2G_05912	3	779	10	22 - 23
AFL2G_03066	3	768	6	19 - 20
AFL2G_11300	3	836	5	Absent
AFL2G_09413	3	909	3	Absent
AFL2G_12245	3	775	4	18 - 19
AFL2G_07763	3	820	1	22 - 23
AFL2G_06408	3	764	2	15 - 16
AFL2G_04928	3	905	2	26 - 27
AFL2G_08626	1	502	1	Absent
AFL2G_02496	1	466	4	Absent
AFL2G_01582	3	807	1	19 - 20

AFL2G_05886	3	839	1	Absent
AFL2G_10164	3	1048	2	Absent
AFL2G_09023	3	815	1	20 -21
AFL2G_11800	3	458	6	Absent
AFL2G_07497	3	752	3	18 – 19
AFL2G_00334	3	1155	2	Absent
AFL2G_9452	3	852	0	Absent
AFL2G_00957	3	797	0	20 – 21
AFL2G_02949	3	761	2	19 - 20
AFL2G_12252	3	776	0	23 – 24
AFL2G_07119	3	634	0	Absent
AFL2G_08111	1	597	0	23 - 24
AFL2G_08686	3	164	2	Absent
AFL2G_02272	3	819	2	Absent
AFL2G_12338	3	908	2	Absent
NFIA_099670	1	616	1	17 - 18
NFIA_060370	3	829	2	Absent
NFIA_080180	3	780	10	18 - 19
NFIA_050080	3	1045	2	Absent
NFIA_027390	3	739	2	21 - 22
NFIA_018950	3	873	8	19 – 20
NFIA_000750	3	860	5	Absent
NFIA_057590	3	864	3	Absent
NFIA_098520	3	790	2	15 - 16
NFIA_010690	1	483	4	Absent
NFIA_060550	1	529	1	Absent
NFIA_064710	1	488	1	Absent

NFIA_009040	1	497	2	Absent
NFIA_054350	3	869	2	19 - 20
NFIA_080070	3	838	2	Absent
NFIA_095760	3	806	1	20 - 21
NFIA_007920	3	769	6	22 - 23
NFIA_112660	3	856	0	Absent
NFIA_057910	3	884	2	Absent
NFIA_100430	3	817	1	20 – 21
NFIA_003180	3	771	0	25 - 26
NFIA_008470	3	297	1	Absent
AO090010000034	3	848	3	Absent
AO090701000274	3	779	10	22 - 23
AO090003001511	3	856	2	Absent
AO090103000127	3	796	3	18 - 19
AO090012000135	3	768	6	19 - 20
AO090009000356	3	861	5	19 -20
AO090009000554	3	964	3	Absent
AO090038000223	3	815	1	20 - 21
AO090166000090	3	841	2	Absent
AO090113000148	1	506	1	Absent
AO090001000544	3	866	2	20 - 21
AO090701000244	3	839	1	Absent
AO090003000497	1	438	3	Absent
AO090166000048	3	852	0	Absent
AO090005000337	3	726	0	Absent
AO090701000841	3	764	2	15 – 16
AO090038000425	3	820	1	22 – 23

AO090001000266	3	752	3	18 – 19
AO090012000003	3	1207	6	Absent
AO090011000140	3	822	1	26 – 27
AO090103000120	3	797	1	Absent
AO090026000123	3	656	1	Absent
AO090005000986	3	798	0	20 – 21
AO090120000075	1	458	1	Absent
AO090103000019	3	961	3	Absent
AO090003000741	3	741	1	Absent
ACLA_019180	1	485	3	Absent
ACLA_010340	3	780	10	24 - 25
ACLA_010450	3	838	2	Absent
ACLA_096980	3	1050	3	Absent
ACLA_028810	3	867	8	18 - 19
ACLA_020660	1	483	4	Absent
ACLA_040420	1	441	5	Absent
ACLA_087610	3	829	2	Absent
ACLA_007810	3	360	4	Absent
ACLA_083710	3	785	3	Absent
ACLA_062400	3	803	0	Absent
ACLA_064280	1	122	1	19 - 20
gw1_4.1698	1	444	1	Absent
e_gw1_6.57	3	1022	3	Absent
e_gw1_4.110	3	765	6	19 - 20
est_GWPlus_C_41054	3	865	2	21 – 22
fge1_pm_C_16000063	3	786	10	21 – 22
fge1_pg_C_14000156	3	816	2	Absent

est_GWPlus_C_140093	1	483	4	Absent
est_fge1_pm_C_100081	3	860	6	19 - 20
e_gw1_6.678	3	815	1	22 – 23
fge1_pg_C_3000425	3	750	3	18 – 19
gw1_15.286	3	826	4	Absent
e_gw1_3.145	3	865	0	Absent
fge1_pg_C_5000054	3	798	3	18 – 19
gw1_13.485	1	496	0	Absent

Appendix 29: Plasmid map. The synthetic gene NFIA_027390

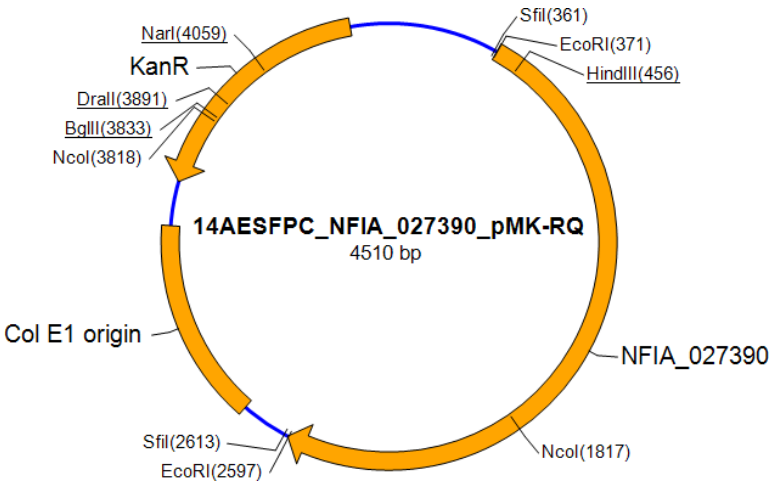


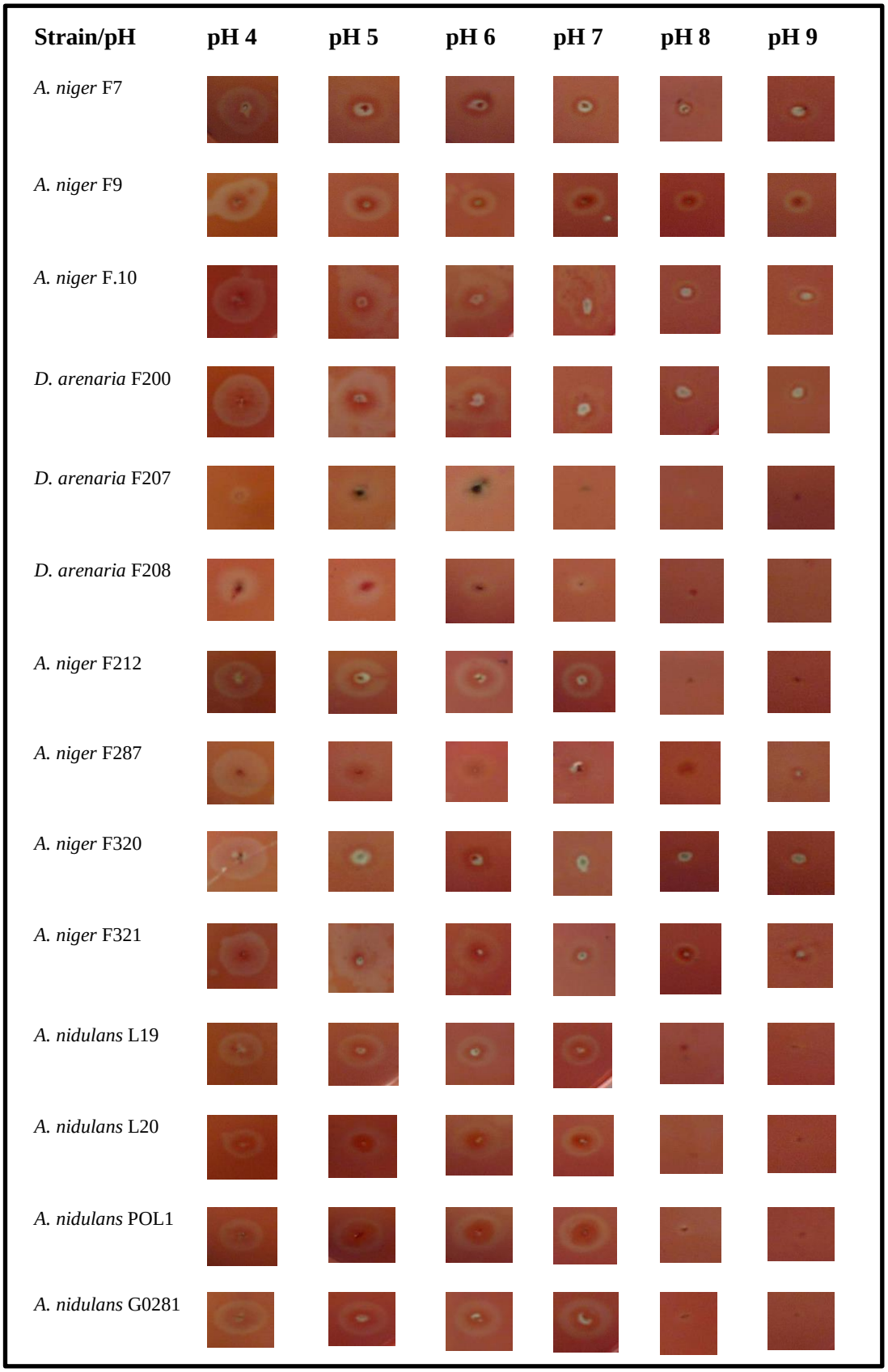
Figure 6.16: Plasmid map. The synthetic gene NFIA_027390 was assembled from synthetic oligonucleotides and/or PCR products. The fragment was cloned into pMK-RQ (kanR) using SfiI and SfiI cloning sites. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100%. 5 µg of the plasmid preparation were lyophilized for shipping (life Technologies).

Appendix 30: Effect of Temperature (^{o}C) on fungal cellulase activity (mm)

Strain/T (^{o}C)	25	30	35	40	45	50
A. niger F.7	1.39 ± 0.03	1.53 ± 0.05	1.63 ± 0.09	1.67 ± 0.05	1.79 ± 0.03	0.82 ± 0.04
A. niger F.9	1.20 ± 0.0	1.21 ± 0.03	1.36 ± 0.05	1.24 ± 0.09	1.53 ± 0.05	0.80 ± 0.0
A. niger F.10	0.00	0.00	0.00	0.00	0.00	0.00
D. arenaria F200	0.00	0.00	0.00	0.00	0.00	0.00
D. salina F201	0.00	0.00	0.00	0.00	0.00	0.00
D. salina F202	0.00	0.00	0.00	0.00	0.00	0.00
D. salina F203	0.00	0.00	0.00	0.00	0.00	0.00
D. salina F204	1.10 ± 0.0	1.19 ± 0.03	1.40 ± 0.05	1.42 ± 0.04	1.84 ± 0.07	1.27 ± 0.05
D. arenaria F205	0.00	0.00	0.00	0.00	0.00	0.00
D. arenaria F206	0.00	0.00	0.00	0.00	0.00	0.00
D. arenaria F207	0.00	0.00	0.00	0.00	0.00	0.00
D. arenaria F208	0.00	0.00	0.00	0.00	0.00	0.00
D. salina F210	0.00	0.00	0.00	0.00	0.00	0.00
A. niger F212	0.00	0.00	0.00	0.91 ± 0.00	0.90 ± 0.00	0.00
A. niger F287	0.80 ± 0.0	0.88 ± 0.04	1.02 ± 0.04	0.00	0.00	0.00
A. niger F320	1.41 ± 0.03	1.57 ± 0.05	1.68 ± 0.04	1.77 ± 0.05	1.49 ± 0.06	0.00
A. niger F321	1.92 ± 0.04	2.04 ± 0.09	2.22 ± 0.04	2.34 ± 0.07	2.21 ± 0.03	0.91 ± 0.03
*A. nidulans*L19	0.00	0.00	0.00	0.00	0.00	0.00
*A. nidulans*L20	0.00	0.00	0.00	0.00	0.00	0.00
*A. nidulans*POL1	0.00	1.21 ± 0.03	1.00 ± 0.0	1.12 ± 0.04	1.21 ± 0.03	0.00
*A. nidulans*GO281	0.00	0.00	0.00	0.00	0.00	0.00

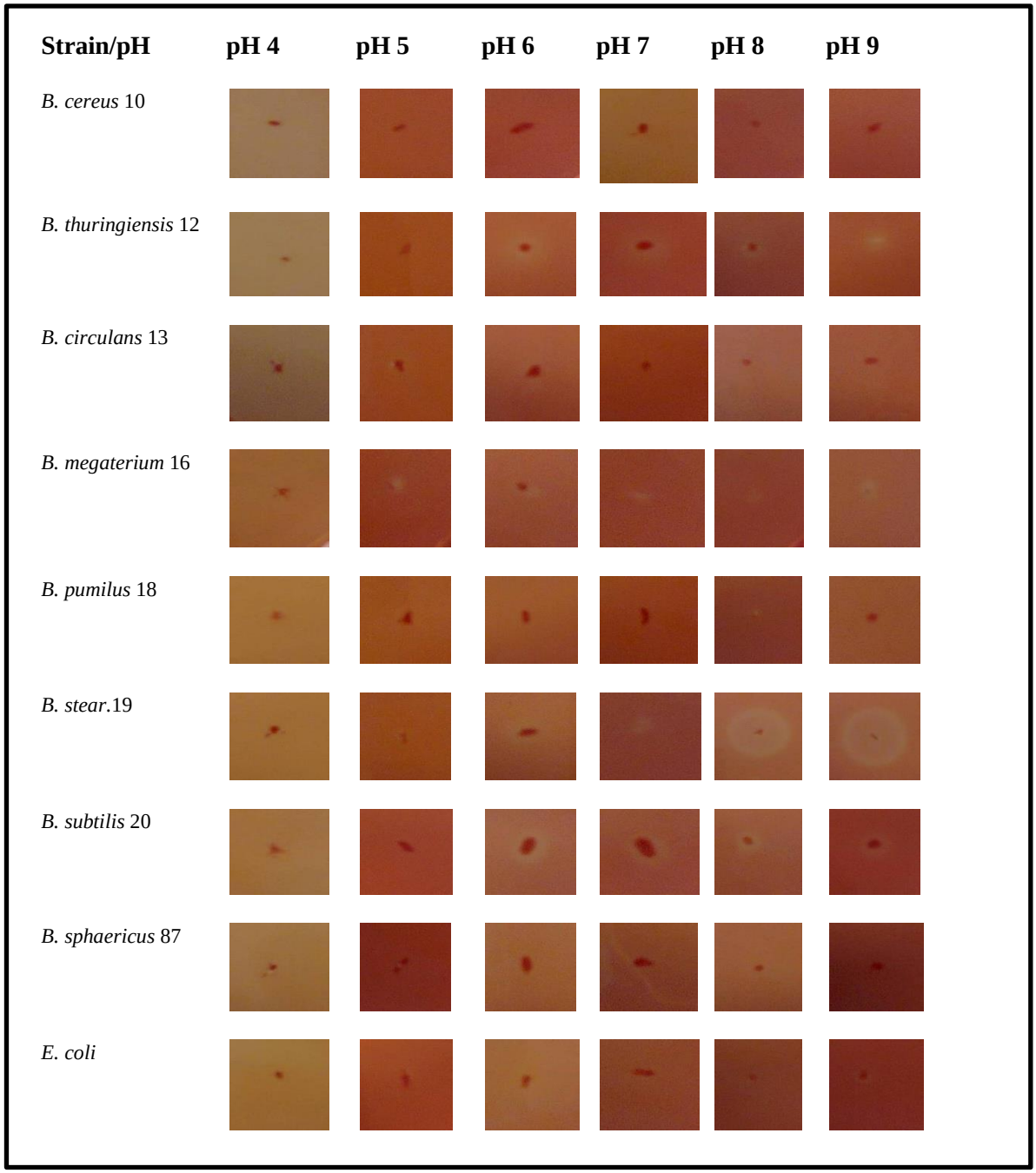
Results are mean values of three replicates. ± = Standard deviation

Appendix 31: Effect of pH profile on fungal crude cellulase activity



Pictures were taken after 24 hours of incubation.

Appendix 32: pH profile of bacterial cellulase using minimal medium and CMC as a carbon source at 35 °C.



Pictures were taken after 72 hours of incubation. *B – Bacillus, B. stear. - B. stearothermophilus, E - Escherichia*

Appendix 33: Effect of carbon sources on fungal growth after 48 hours of incubation on minimal medium

Strain	NCS *	Glucose	CMC[x]	Cellobiose	Cellulose^
A. niger F7	-	++++	-	+	-
A. niger F9	-	+++	-	+	-
A. niger F10	-	++++	-	+	-
D. arenaria F200	-	+++	+	+	++
D. arenaria F207	-	+++	+	++	++++
D. arenaria F208	-	+++	+	++	++
A. niger F212	-	++++	++	++	+++
A. niger F.287	-	++++	-	+	-
A. niger F.320	-	++++	-	+	-
A. niger F321	-	++++	-	+	+
A. nidulans L19	-	++++	++	+++	++
A. nidulans L20	-	++++	+	++	+
A. nidulans POL1	-	++++	++	++++	+++
A. nidulans G0281	-	++++	++	++++	+

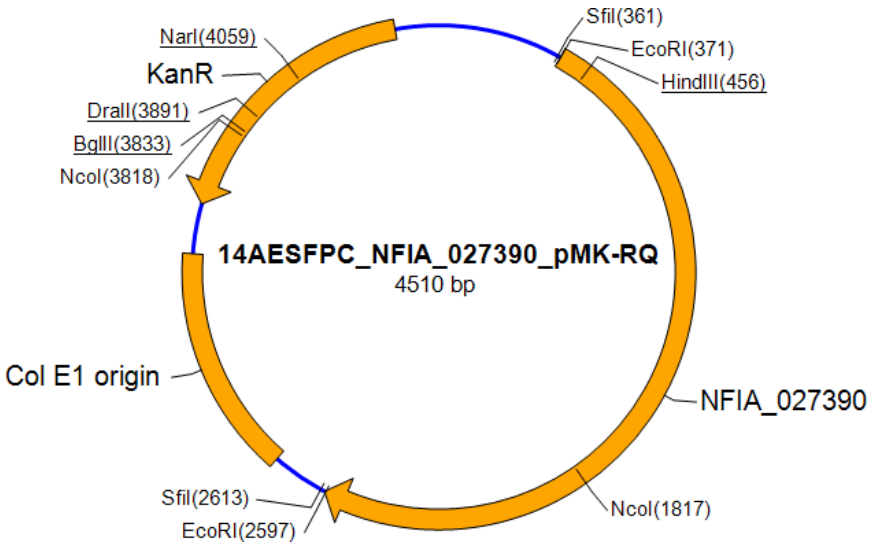
Growth score: -: no growth; +: very poor growth; ++: poor growth; +++: moderate growth; ++++: good growth. *No carbon source; [x]Carboxymethyl cellulose. ^cellulose plates were incubated for six days. See Figure 4.7 for example photo.

Appendix 34: Effect of Temperature (^{o}C) on crude cellulase activity of selected *Bacillus* strains (mm)

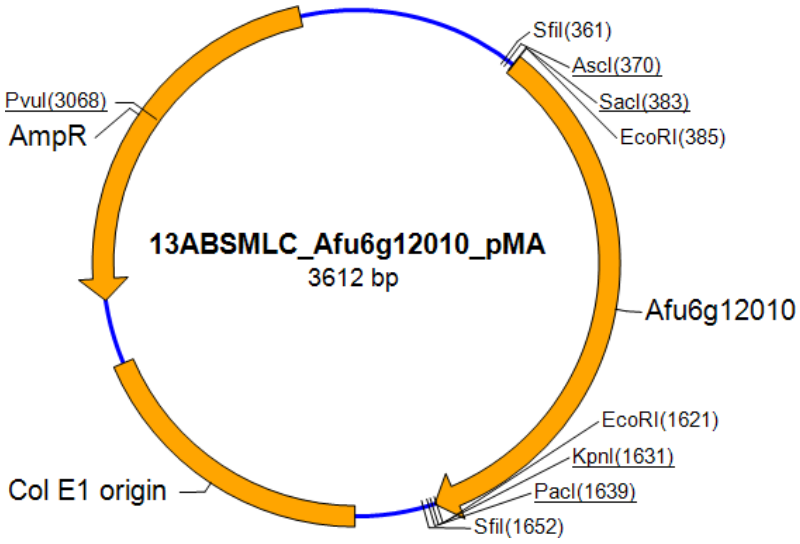
Strain/T (^{o}C)	25	30	35	40	45	50
B. cereus	0.00	0.00	0.00	0.00	0.00	0.00
B. megaterium	0.00	0.00	0.00	0.00	0.00	0.00
B. thuringiensis	0.90 ± 0.06	1.13 ± 0.06	1.40 ± 0.10	1.40 ± 0.10	0.00	0.00
B. circulans	0.00	0.77 ± 0.06	1.03 ± 0.06	0.00	0.00	0.00
B. subtilis	1.10 ± 0.0	1.67 ± 0.06	1.97 ± 0.06	2.97 ± 0.06	3.90 ± 0.17	2.13 ± 0.06
B. pumilus	0.00	0.00	0.00	0.00	0.00	0.00
B. sphaericus	0.00	0.00	0.00	0.00	0.00	0.00

Results are mean values of three replicates. ± = Standard deviation

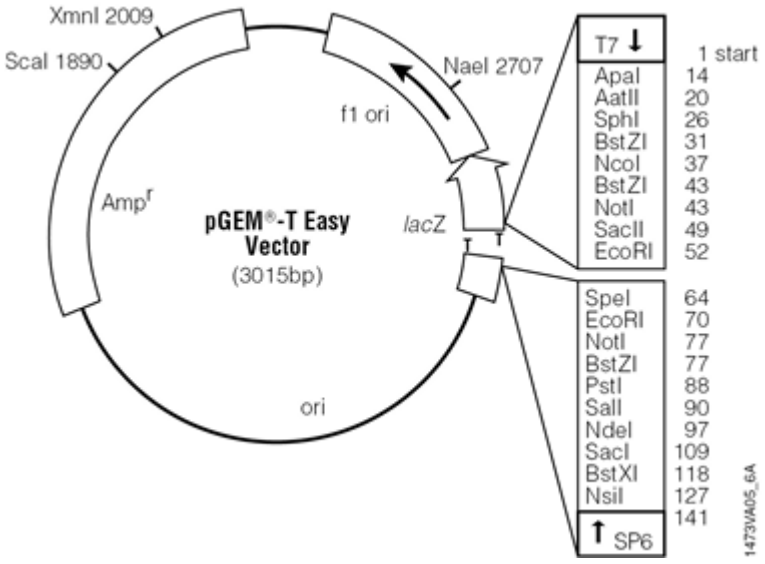
Appendix 35: NFIA_027390 plasmid DNA within cloning vector pMK (Invitrogen, 2014)



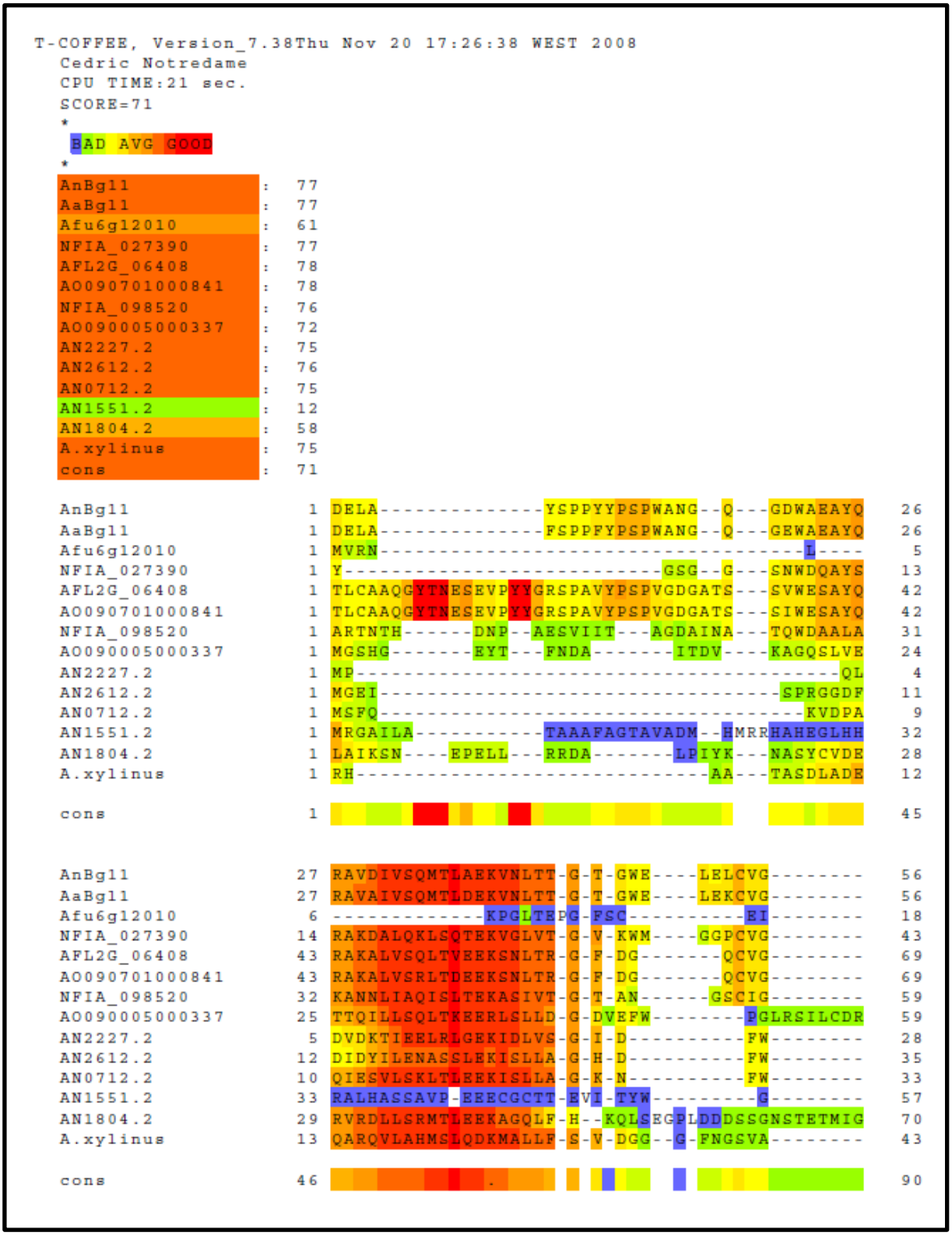
Appendix 36: Synthetic gene Afu6g12010 within cloning vector pMA (Invitrogen, 2013)

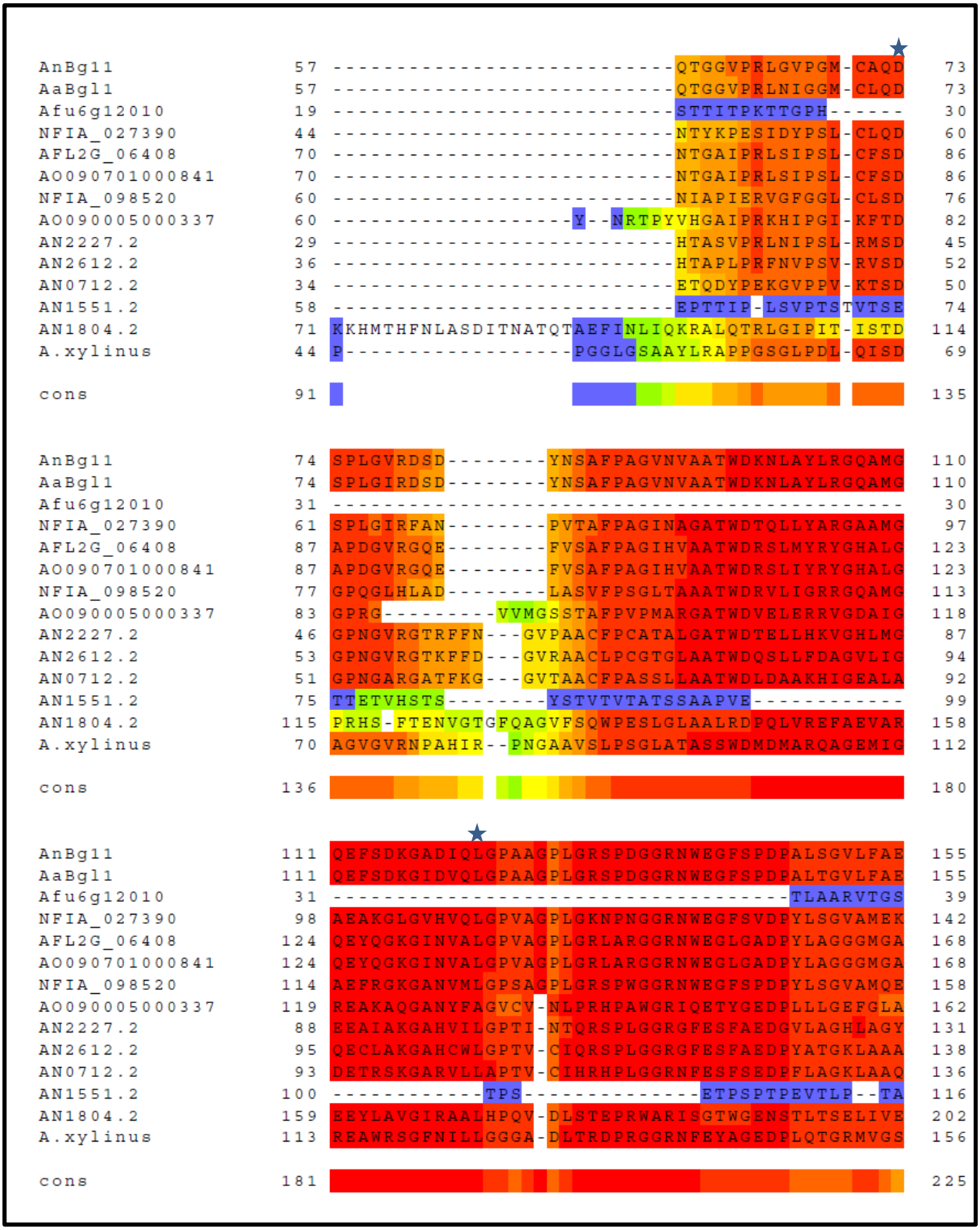


Appendix 37: Restriction map of T/A cloning vector (pGEM®-T Easy Vector)



Appendix 38: Multiple sequence alignment of selected β-glucosidases

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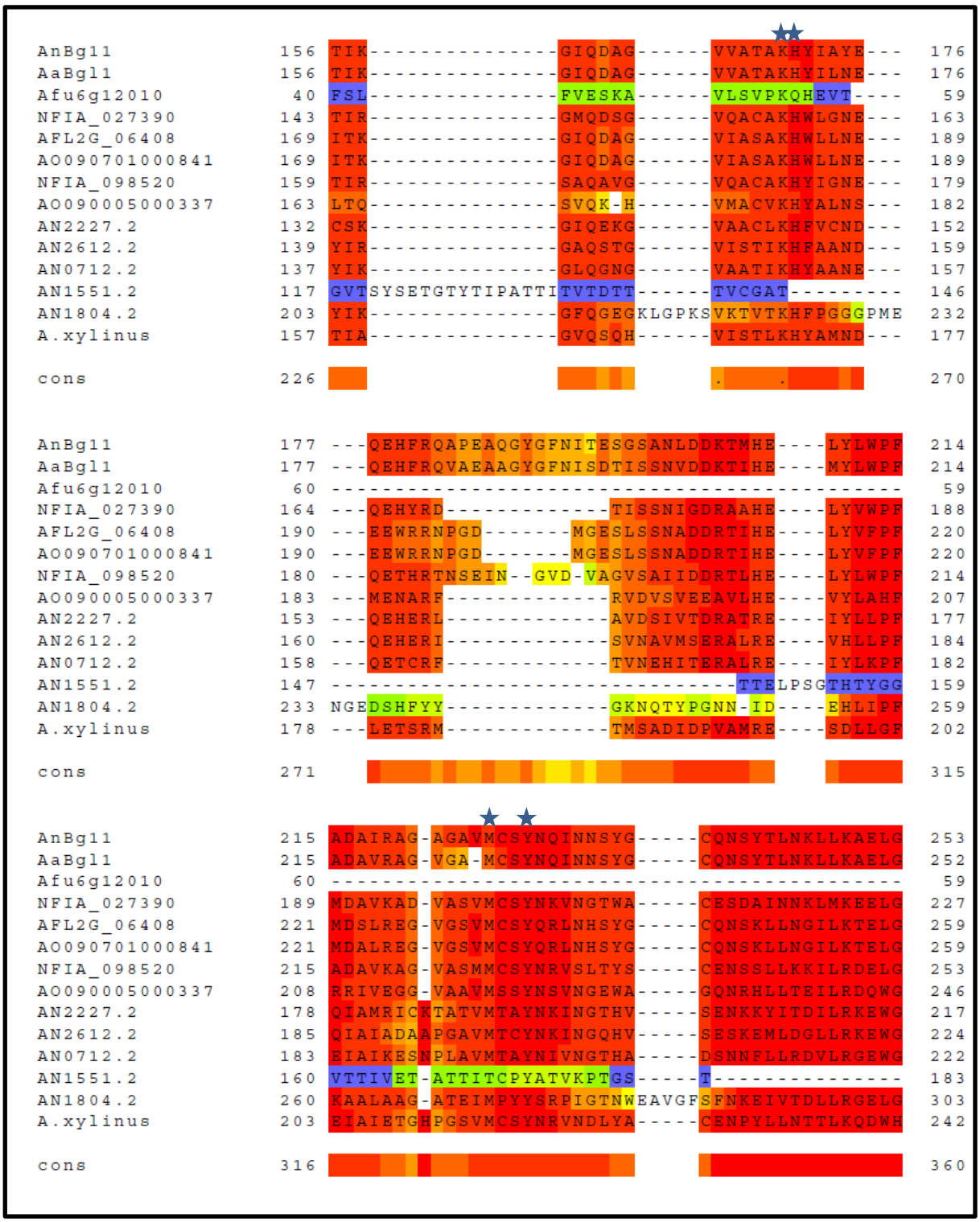
AaBg11 57 ----------QTGGVPRLNIGGM-CLQD 73
Afu6g12010 19 ----------STTITPKTTGPH------ 30
NFIA_027390 44 ----------NTYKPESIDYPSL-CLQD 60
AFL2G_06408 70 ----------NTGAIPRLSIPSL-CFSD 86
AO090701000841 70 ----------NTGAIPRLSIPSL-CFSD 86
NFIA_098520 60 ----------NIAPIERVGFGGL-CLSD 76
AO090005000337 60 ----------Y--NRTPYVHGAIPRKHIPGI-KFTD 82
AN2227.2 29 ----------HTASVPRLNIPSL-RMSD 45
AN2612.2 36 ----------HTAPLPRFNVPSV-RVSD 52
AN0712.2 34 ----------ETQDYPEKGVPPV-KTSD 50
AN1551.2 58 ----------EPTTIP-LSVPTSTVTSE 74
AN1804.2 71 KKHMTHFNLASDITNATQTAEFINLIQKRALQTRLGIPIT-ISTD 114
A.xylinus 44 P----------PGGLGSAAYLRAPPGSGLPDL-QISD 69
cons 91 135
AnBg11 74 SPLGVRDSD--------YNSAFPAGVNVAATWDKNLAYLRGQAMG 110
AaBg11 74 SPLGIRDSD--------YNSAFPAGVNVAATWDKNLAYLRGQAMG 110
Afu6g12010 31 ---------- 30
NFIA_027390 61 SPLGIRFAN--------PVTAFPAGINAGATWDTQLLYARGAAMG 97
AFL2G_06408 87 APDGVRGQE--------FVSAFPAGIHVAATWDRSLMYRYGHALG 123
AO090701000841 87 APDGVRGQE--------FVSAFPAGIHVAATWDRSLIYRYGHALG 123
NFIA_098520 77 GPQGLHLAD--------LASVFPSGLTAAATWDRVLIGRRGQAMG 113
AO090005000337 83 GPRG---------VVMGSSTAFPVPMARGATWDVELERRVGDAIG 118
AN2227.2 46 GPNGVRGTRFFN---GVPAACFPCATALGATWDTELLHKVGHLMG 87
AN2612.2 53 GPNGVRGTKFFD---GVRAACLPCGTGLAATWDQSLLFDAGVLIG 94
AN0712.2 51 GPNGARGATFKG---GVTAACFPASSLLAATWDLDAAKHIGEALA 92
AN1551.2 75 TTETVHSTS--------YSTVTVTATSSAAPVE---------- 99
AN1804.2 115 PRHS-FTENVGTGFQAGVFSQWPESLGLAALRDPQLVREFAEVAR 158
A.xylinus 70 AGVGVRNPAHIR--PNGAAVSLPSGLATASSWDMDMARQAGEMIG 112
cons 136 180
AnBg11 111 QEFSDKGADIQLGPAAGPLGRSPDGGRNWEGFSPDPALSGVLFAE 155
AaBg11 111 QEFSDKGIDVQLGPAAGPLGRSPDGGRNWEGFSPDPALTGVLFAE 155
Afu6g12010 31 ----------TLAARVTGS 39
NFIA_027390 98 AEAKGLGVHVQLGPVAGPLGKNPNGGRNWEGFSVDPYLSGVAMEK 142
AFL2G_06408 124 QEYQGKGINVALGPVAGPLGRLARGGRNWEGLGADPYLAGGGMGA 168
AO090701000841 124 QEYQGKGINVALGPVAGPLGRLARGGRNWEGLGADPYLAGGGMGA 168
NFIA_098520 114 AEFRGKGANVMLGPSAGPLGRSPWGGRNWEGFSPDPYLSGVAMQE 158
AO090005000337 119 REAKAQGANYFAGVCV-NLPRHPAWGRIQETYGEDPLLLGEFGLA 162
AN2227.2 88 EEAIAKGAHVILGPTI-NTQRSPLGGRGFESFAEDGVLAGHLAGY 131
AN2612.2 95 QECLAKGAHCWLGPTV-CIQRSPLGGRGFESFAEDPYATGKLAAA 138
AN0712.2 93 DETRSKGARVLLAPTV-CIHRHPLGGRNFESFSEDPFLAGKLAAQ 136
AN1551.2 100 ----------TPS----------ETPSPTPEVTLP--TA 116
AN1804.2 159 EEYLAVGIRAALHPQV-DLSTEPRWARISGTWGENSTLTSELIVE 202
A.xylinus 113 REAWRSGFNILLGGGA-DLTRDPRGGRNFEYAGEDPLQTGRMVGS 156
cons 181 225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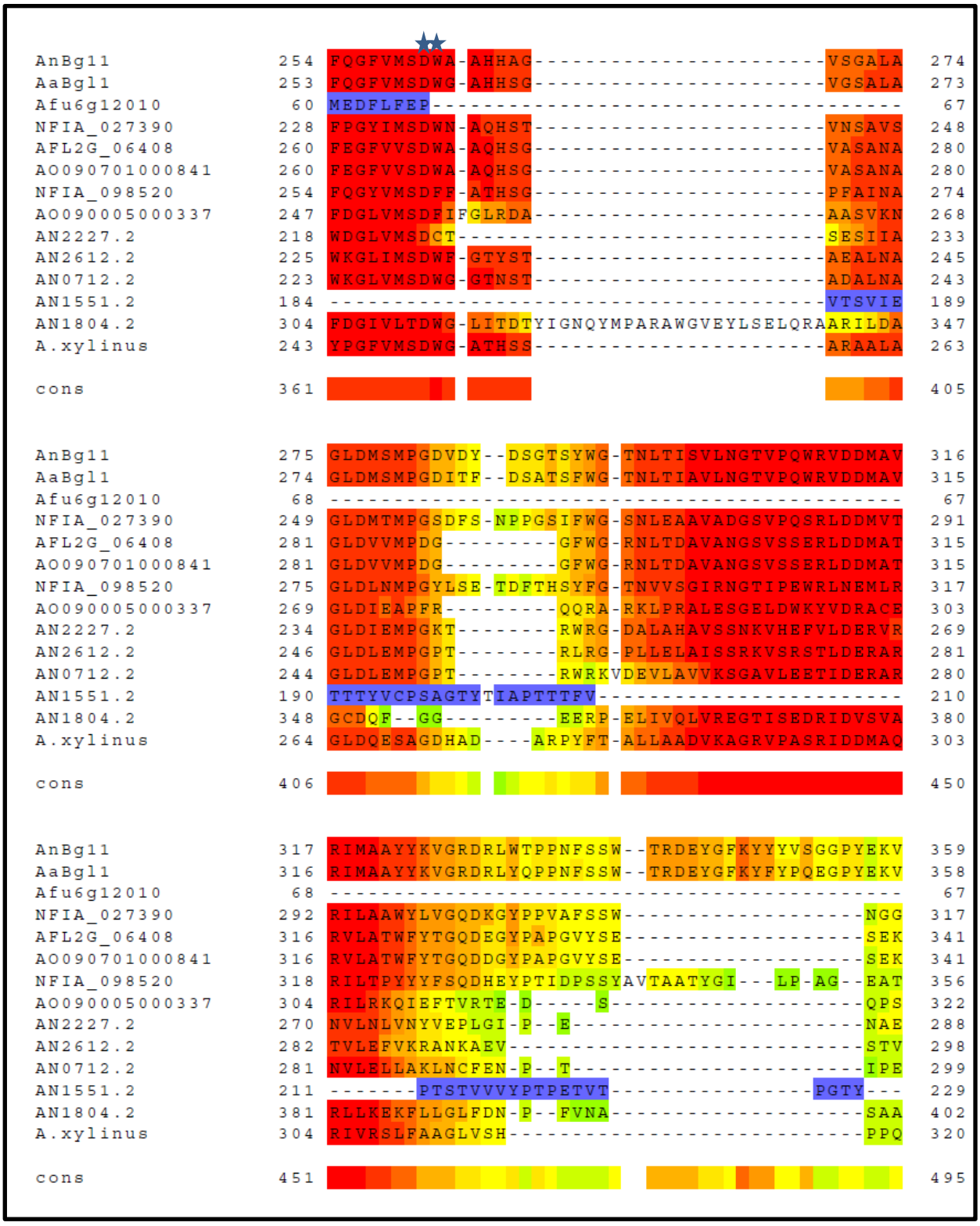
AaBg11 156 TIK----------GIQDAG------VVATAKHYILNE--- 176
Afu6g12010 40 FSL----------FVESKA------VLSVPKQHEVT---- 59
NFIA_027390 143 TIR----------GMQDSG------VQACAKHWLGNE--- 163
AFL2G_06408 169 ITK----------GIQDAG------VIASAKHWLLNE--- 189
AO090701000841 169 ITK----------GIQDAG------VIASAKHWLLNE--- 189
NFIA_098520 159 TIR----------SAQAVG------VQACAKHYIGNE--- 179
AO090005000337 163 LTQ----------SVQK-H------VMACVKHYALNS--- 182
AN2227.2 132 CSK----------GIQEKG------VAACLKHFVCND--- 152
AN2612.2 139 YIR----------GAQSTG------VISTIKHFAAND--- 159
AN0712.2 137 YIK----------GLQGNG------VAATIKHYAANE--- 157
AN1551.2 117 GVTSYSETGTYTIPATTITVTDTT------TVCGAT---------- 146
AN1804.2 203 YIK----------GFQGEGKLGPKSVKTVTKHFPGGGPME 232
A.xylinus 157 TIA----------GVQSQH------VISTLKHYAMND--- 177
cons 226 270
AnBg11 177 ---QEHFRQAPEAQGYGFNITESGSANLDDKTMHE----LYLWPF 214
AaBg11 177 ---QEHFRQVAEAAGYGFNISDTISSNVDDKTIHE----MYLWPF 214
Afu6g12010 60 59
NFIA_027390 164 ---QEHYRD----------TISSNIGDRAAHE----LYVWPF 188
AFL2G_06408 190 ---EEWRRNPGD-------MGESLSSNADDRTIHE----LYVFPF 220
AO090701000841 190 ---EEWRRNPGD-------MGESLSSNADDRTIHE----LYVFPF 220
NFIA_098520 180 ---QETHRTNSEIN--GVD-VAGVSAIIDDRTLHE----LYLWPF 214
AO090005000337 183 ---MENARF----------RVDVSVEEAVLHE----VYLAHF 207
AN2227.2 153 ---QEHERL----------AVDSIVTDRATRE----IYLLPF 177
AN2612.2 160 ---QEHERI----------SVNAVMSERALRE----VHLLPF 184
AN0712.2 158 ---QETCRF----------TVNEHITERALRE----IYLKPF 182
AN1551.2 147 ----------TTELPSGTHTYGG 159
AN1804.2 233 NGEDSHFYY----------GKNQTYPGNN-ID----EHLIPF 259
A.xylinus 178 ---LETSRM----------TMSADIDPVAMRE----SDLLGF 202
cons 271 315
AnBg11 215 ADAIRAG-AGAVMCSYNQINNSYG-----CQNSYTLNKLLKAELG 253
AaBg11 215 ADAVRAG-VGA-MCSYNQINNSYG-----CQNSYTLNKLLKAELG 252
Afu6g12010 60 59
NFIA_027390 189 MDAVKAD-VASVMCSYNKVNGTWA-----CESDAINNKLMKEELG 227
AFL2G_06408 221 MDSLREG-VGSVMCSYQRLNHSYG-----CQNSKLLNGILKTELG 259
AO090701000841 221 MDALREG-VGSVMCSYQRLNHSYG-----CQNSKLLNGILKTELG 259
NFIA_098520 215 ADAVKAG-VASMMCSYNRVSLTYS-----CENSSLLKKILRDELG 253
AO090005000337 208 RRIVEGG-VAAVMSSYNSVNGEWA-----GQNRHLLTEILRDQWG 246
AN2227.2 178 QIAMRICKTATVMTAYNKINGTHV-----SENKKYITDILRKEWG 217
AN2612.2 185 QIAIADAAPGAVMTCYNKINGQHV-----SESKEMLDGLLRKEWG 224
AN0712.2 183 EIAIKESNPLAVMTAYNIVNGTHA-----DSNNFLLRDVLRGEWG 222
AN1551.2 160 VTTIVET-ATTITCPYATVKPTGS-----T---------------- 183
AN1804.2 260 KAALAAG-ATEIMPYYSRPIGTNWEAVGFSFNKEIVTDLLRGELG 303
A.xylinus 203 EIAIETGHPGSVMCSYNRVNDLYA-----CENPYLLNTTLKQDWH 242
cons 316 360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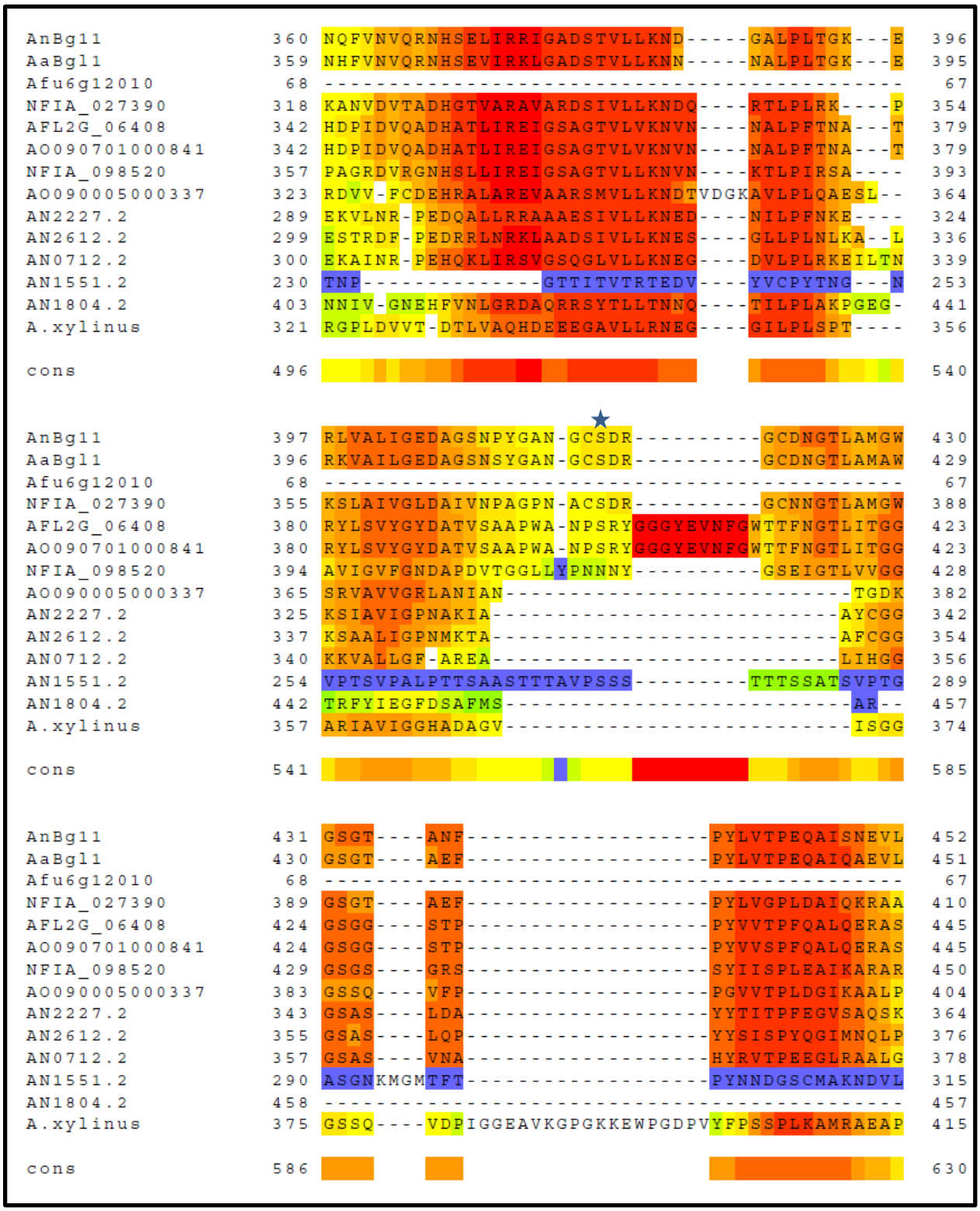
AaBg11 253 FQGFVMSDWG-AHHSG----------VGSALA 273
Afu6g12010 60 MEDFLFEP---------- 67
NFIA_027390 228 FPGYIMSDWN-AQHST----------VNSAVS 248
AFL2G_06408 260 FEGFVVSDWA-AQHSG----------VASANA 280
AO090701000841 260 FEGFVVSDWA-AQHSG----------VASANA 280
NFIA_098520 254 FQGYVMSDFF-ATHSG----------PFAINA 274
AO090005000337 247 FDGLVMSDFIFGLRDA----------AASVKN 268
AN2227.2 218 WDGLVMSDCT----------SESIIA 233
AN2612.2 225 WKGLIMSDWF-GTYST----------AEALNA 245
AN0712.2 223 WKGLVMSDWG-GTNST----------ADALNA 243
AN1551.2 184 ----------VTSVIE 189
AN1804.2 304 FDGIVLTDWG-LITDTYIGNQYMPARAWGVEYLSELQRAARILDA 347
A.xylinus 243 YPGFVMSDWG-ATHSS----------ARAALA 263
cons 361 405
AnBg11 275 GLDMSMPGDVDY--DSGTSYWG-TNLTISVLNGTVPQWRVDDMAV 316
AaBg11 274 GLDMSMPGDITF--DSATSFWG-TNLTIAVLNGTVPQWRVDDMAV 315
Afu6g12010 68 ---------- 67
NFIA_027390 249 GLDMTMPGSDFS-NPPGSIFWG-SNLEAAVADGSVPQSRLDDMVT 291
AFL2G_06408 281 GLDVVMPDG---------GFWG-RNLTDAVANGSVSSERLDDMAT 315
AO090701000841 281 GLDVVMPDG---------GFWG-RNLTDAVANGSVSSERLDDMAT 315
NFIA_098520 275 GLDLNMPGYLSE-TDFTHSYFG-TNVVSGIRNGTIPEWRLNEMLR 317
AO090005000337 269 GLDIEAPFR---------QQRA-RKLPRALESGELDWKYVDRACE 303
AN2227.2 234 GLDIEMPGKT--------RWRG-DALAHAVSSNKVHEFVLDERVR 269
AN2612.2 246 GLDLEMPGPT--------RLRG-PLLELAISSRKVSRSTLDERAR 281
AN0712.2 244 GLDLEMPGPT--------RWRKVDEVLAVVKSGAVLEETIDERAR 280
AN1551.2 190 TTTYVCPSAGTYTIAPTTTFV---------- 210
AN1804.2 348 GCDQF--GG---------EERP-ELIVQLVREGTISEDRIDVSVA 380
A.xylinus 264 GLDQESAGDHAD----ARPYFT-ALLAADVKAGRVPASRIDDMAQ 303
cons 406 450
AnBg11 317 RIMAAYYKVGRDRLWTPPNFSSW--TRDEYGFKYYYVSGGPYEKV 359
AaBg11 316 RIMAAYYKVGRDRLYQPPNFSSW--TRDEYGFKYFYPQEGPYEKV 358
Afu6g12010 68 ---------- 67
NFIA_027390 292 RILAAWYLVGQDKGYPPVAFSSW----------NGG 317
AFL2G_06408 316 RVLATWFYTGQDEGYPAPGVYSE----------SEK 341
AO090701000841 316 RVLATWFYTGQDDGYPAPGVYSE----------SEK 341
NFIA_098520 318 RILTPYYYFSQDHEYPTIDPSSYAVTAATYGI---LP-AG--EAT 356
AO090005000337 304 RILRKQIEFTVRTE-D-----S----------QPS 322
AN2227.2 270 NVLNLVNYVEPLGI-P--E----------NAE 288
AN2612.2 282 TVLEFVKRANKAEV----------STV 298
AN0712.2 281 NVLELLAKLNCFEN-P--T----------IPE 299
AN1551.2 211 -------PTSTVVVYPTPETVT----------PGTY--- 229
AN1804.2 381 RLLKEKFLLGLFDN-P--FVNA----------SAA 402
A.xylinus 304 RIVRSLFAAGLVSH----------PPQ 320
cons 451 495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AaBgl1 359 NHFVNVQRNHSEVIRKLGADSTVLLKNN-----NALPLTGK---E 395
Afu6g12010 68 ---------- 67
NFIA_027390 318 KANVDVTADHGTVARAVARDSIVLLKNDQ----RTLPLRK----P 354
AFL2G_06408 342 HDPIDVQADHATLIREIGSAGTVLVKNVN----NALPFTNA---T 379
AO0907010000841 342 HDPIDVQADHATLIREIGSAGTVLVKNVN----NALPFTNA---T 379
NFIA_098520 357 PAGRDVRGNHSLLIREIGSAGTVLLKNVN----KTLPIRSA---- 393
AO0900005000337 323 RDVV-FCDEHRALAREVAARSMVLLKNDTVDGKAVLPLQAESL-- 364
AN2227.2 289 EKVLNR-PEDQALLRRAAAESIVLLKNED----NILPFNKE---- 324
AN2612.2 299 ESTRDF-PEDRRLNRKLAADSIVLLKNES----GLLPLNLKA--L 336
AN0712.2 300 EKAINR-PEHQKLIRSVGSQGLVLLKNEG----DVLPLRKEILTN 339
AN1551.2 230 TNP----------GTTITVTRTEDV----YVCPYTNG---N 253
AN1804.2 403 NNIV-GNEHFVNLGRDAQRRSYTLLTNNQ----TILPLAKPGEG- 441
A.xylinus 321 RGPLDVVT-DTLVAQHDEEEGAVLLRNEG----GILPLSPT---- 356
cons 496 540
AnBg11 397 RLVALIGEDAGSNPYGAN-GCSDR----------GCDNGTLAMGW 430
AaBgl1 396 RKVAILGEDAGSNSYGAN-GCSDR----------GCDNGTLAMAW 429
Afu6g12010 68 ---------- 67
NFIA_027390 355 KSLAIVGLDAIVNPAGPN-ACSDR----------GCNNGTLAMGW 388
AFL2G_06408 380 RYLSVYGYDATVSAAPWA-NPSRYGGGYEVNFGWTTFNGTLITGG 423
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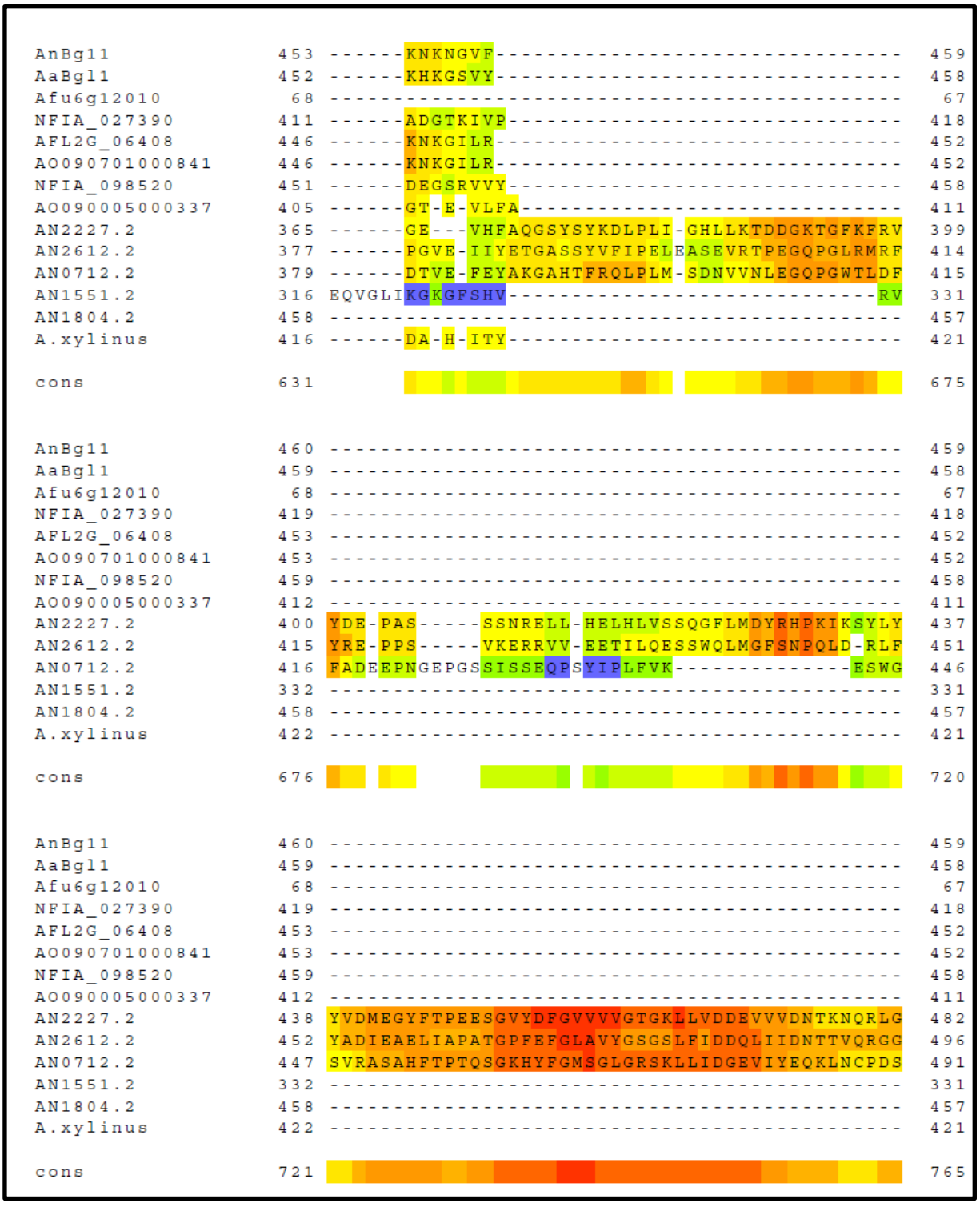
NFIA_098520 394 AVIGVFGNDAPDVTGGLLYPNNNY----------GSEIGTLVVGG 428
AO0900005000337 365 SRVAVVGRLANIAN----------TGDK 382
AN2227.2 325 KSIAVIGPNAKIA----------AYCGG 342
AN2612.2 337 KSAALIGPNMKTA----------AFCGG 354
AN0712.2 340 KKVALLGF-AREA----------LIHGG 356
AN1551.2 254 VPTSVPALPTTSAASTTTAVPSSS---------TTTSSATSVPTG 289
AN1804.2 442 TRFYIEGFDSAFMS----------AR-- 457
A.xylinus 357 ARIAVIGGHADAGV----------ISGG 374
cons 541 585
AnBg11 431 GSGT----ANF----------PYLVTPEQAISNEVL 452
AaBgl1 430 GSGT----AEF----------PYLVTPEQAIQAEVL 451
Afu6g12010 68 ---------- 67
NFIA_027390 389 GSGT----AEF----------PYLVGPLDAIQKRAA 410
AFL2G_06408 424 GSGG----STP----------PYVVTPFQALQERAS 445
AO0907010000841 424 GSGG----STP----------PYVVSPFQALQERAS 445
NFIA_098520 429 GSGS----GRS----------SYIISPLEAIKARAR 450
AO0900005000337 383 GSSQ----VFP----------PGVVTPLDGIKAALP 404
AN2227.2 343 GSAS----LDA----------YYTITPFEGVSAQSK 364
AN2612.2 355 GSAS----LQP----------YYSISPYQGIMNQLP 376
AN0712.2 357 GSAS----VNA----------HYRVTPEEGLRAALG 378
AN1551.2 290 ASGNKMGMTFT----------PYNNDGSCMAKNDVL 315
AN1804.2 458 ---------- 457
A.xylinus 375 GSSQ----VDPIGGEAVKGPGKKEWPGDPVYFPSSPLKAMRAEAP 415
cons 586 630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AaBgl1 452 ------KHKGSVY---------- 458
Afu6g12010 68 ---------- 67
NFIA_027390 411 ------ADGTKIVP---------- 418
AFL2G_06408 446 ------KNKGILR---------- 452
AO090701000841 446 ------KNKGILR---------- 452
NFIA_098520 451 ------DEGSRVVY---------- 458
AO090005000337 405 ------GT-E-VLFA---------- 411
AN2227.2 365 ------GE---VHFAQGSYSYKDLPLI-GHLLKTDDGKTGFKFRV 399
AN2612.2 377 ------PGVE-IIYETGASSYVFIPELEASEVRTPEGQPGLRMRF 414
AN0712.2 379 ------DTVE-FEYAKGAHTFRQLPLM-SDNVVNLEGQPGWTLDF 415
AN1551.2 316 EQVGLIKGKGFSHV----------RV 331
AN1804.2 458 ---------- 457
A.xylinus 416 ------DA-H-ITY---------- 421
cons 631 675
AnBg11 460 ---------- 459
AaBgl1 459 ---------- 458
Afu6g12010 68 ---------- 67
NFIA_027390 419 ---------- 418
AFL2G_06408 453 ---------- 452
AO090701000841 453 ---------- 452
NFIA_098520 459 ---------- 458
AO090005000337 412 ---------- 411
AN2227.2 400 YDE-PAS-----SSNRELL-HELHLVSSQGFLMDYRHPKIKSYLY 437
AN2612.2 415 YRE-PPS-----VKERRVV-EETILQESSWQLMGFSNPQLD-RLF 451
AN0712.2 416 FADEEPNGEPGSSISSEQPSYIPLFVK----------ESWG 446
AN1551.2 332 ---------- 331
AN1804.2 458 ---------- 457
A.xylinus 422 ---------- 421
cons 676 720
AnBg11 460 ---------- 459
AaBgl1 459 ---------- 458
Afu6g12010 68 ---------- 67
NFIA_027390 419 ---------- 418
AFL2G_06408 453 ---------- 452
AO090701000841 453 ---------- 452
NFIA_098520 459 ---------- 458
AO090005000337 412 ---------- 411
AN2227.2 438 YVDMEGYFTPEESGVYDFGVVVVGTGKLLVDDEVVVDNTKNQRLG 482
AN2612.2 452 YADIEAELIAPATGPFEFGLAVYGSGSLFIDDQLIIDNTTVQRGG 496
AN0712.2 447 SVRASAHFTPTQSGKHYFGMSGLGRSKLLIDGEVIYEQKLNCPDS 491
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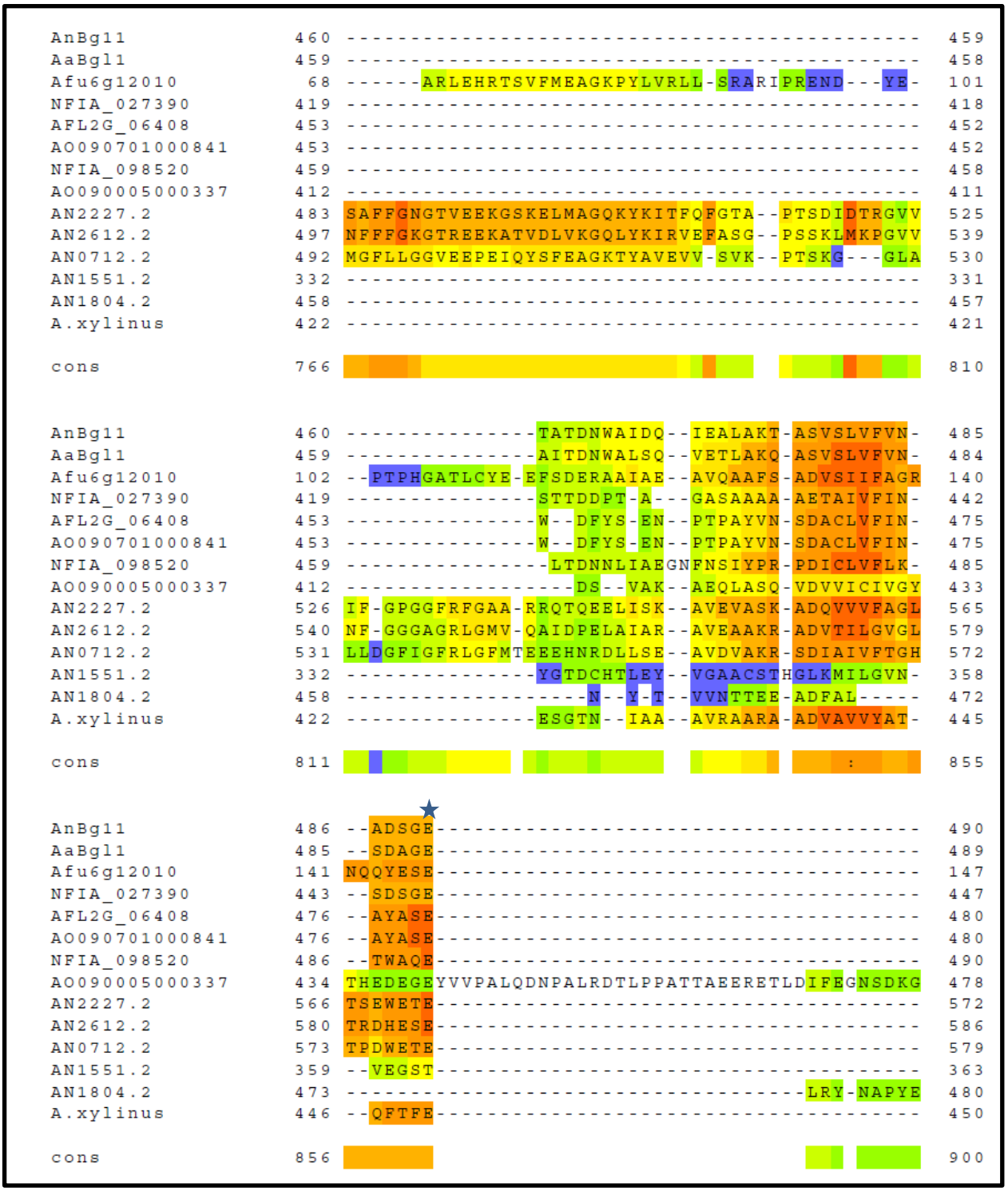
AN1804.2 458 ---------- 457
A.xylinus 422 ---------- 421
cons 721 765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AaBg11 459 ---------- 458
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AFL2G_06408 453 ---------- 452
AO090701000841 453 ---------- 452
NFIA_098520 459 ---------- 458
AO090005000337 412 ---------- 411
AN2227.2 483 SAFFGNGTVEEKGSKELMAGQKYKITFQFGTA--PTSDIDTRGVV 525
AN2612.2 497 NFFFGKGTREEKATVDLVKGQLYKIRVEFASG--PSSKLMKPGVV 539
AN0712.2 492 MGFLLGGVEEPEIQYSFEAGKTYAVEVV-SVK--PTSKG---GLA 530
AN1551.2 332 ---------- 331
AN1804.2 458 ---------- 457
A.xylinus 422 ---------- 421
cons 766 810
AnBg11 460 ----------TATDNWAIDQ--IEALAKT-ASVSLVFVN- 485
AaBg11 459 ----------AITDNWALSQ--VETLAKQ-ASVSLVFVN- 484
Afu6g12010 102 --PTPHGATLCYE-EFSDERAAIAE--AVQAAFS-ADVSIIFAGR 140
NFIA_027390 419 ----------STTDDPT-A---GASAAAA-AETAIVFIN- 442
AFL2G_06408 453 ----------W--DFYS-EN--PTPAYVN-SDACLVFIN- 475
AO090701000841 453 ----------W--DFYS-EN--PTPAYVN-SDACLVFIN- 475
NFIA_098520 459 ----------LTDNNLIAEGNFNSIYPR-PDICLVFLK- 485
AO090005000337 412 ----------DS--VAK--AEQLASQ-VDVVICIVGY 433
AN2227.2 526 IF-GPGGFRFGAA-RRQTQEELISK--AVEVASK-ADQVVVFAGL 565
AN2612.2 540 NF-GGGAGRLGMV-QAIDPELAIAR--AVEAAKR-ADVTILGVGL 579
AN0712.2 531 LLDGFIGFRLGFMTEEEHNRDLLSE--AVDVAKR-SDIAIVFTGH 572
AN1551.2 332 ----------YGTDCHTLEY--VGAACSTHGLKMILGVN- 358
AN1804.2 458 ----------N--Y-T--VVNTTEE-ADFAL----- 472
A.xylinus 422 ----------ESGTN--IAA--AVRAARA-ADVAVVYAT- 445
cons 811 855
AnBg11 486 --ADSGE---------- 490
AaBg11 485 --SDAGE---------- 489
Afu6g12010 141 NQQYESE---------- 147
NFIA_027390 443 --SDSGE---------- 447
AFL2G_06408 476 --AYASE---------- 480
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NFIA_098520 486 --TWAQE---------- 490
AO090005000337 434 THEDEGEYVVPALQDNPALRDTLPPATTAEERETLDIFEGNSDKG 478
AN2227.2 566 TSEWETE---------- 572
AN2612.2 580 TRDHESE---------- 586
AN0712.2 573 TPDWETE---------- 579
AN1551.2 359 --VEGST---------- 363
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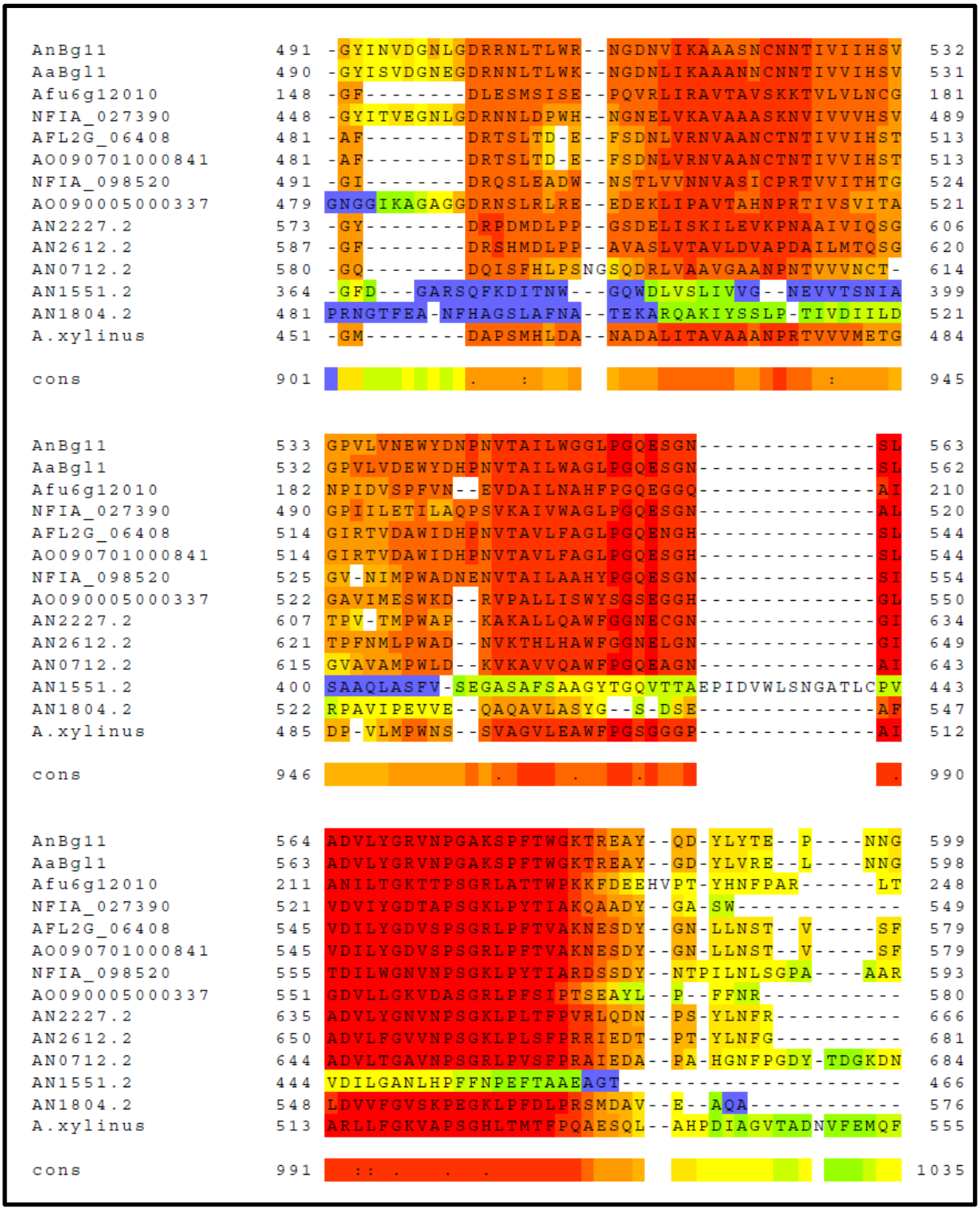
cons 856 900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Afu6g12010 148 -GF--------DLESMSISE--PQVRLIRAVTAVSKKTVLVLNCG 181
NFIA_027390 448 -GYITVEGNLGDRNNLDPWH--NGNELVKAVAAASKNVIVVVHSV 489
AFL2G_06408 481 -AF--------DRTSLTD-E--FSDNLVRNVAANCTNTIVVIHST 513
AO090701000841 481 -AF--------DRTSLTD-E--FSDNLVRNVAANCTNTIVVIHST 513
NFIA_098520 491 -GI--------DRQSLEADW--NSTLVVNNVASICPRTVVITHTG 524
AO090005000337 479 GNGGIKAGAGGDRNSLRLRE--EDEKLIPAVTAHNPRTIVSVITA 521
AN2227.2 573 -GY--------DRPDMDLPP--GSDELISKILEVKPNAAIVIQSG 606
AN2612.2 587 -GF--------DRSHMDLPP--AVASLVTAVLDVAPDAILMTQSG 620
AN0712.2 580 -GQ--------DQISFHLPSNGSQDRLVAAVGAANPNTVVVNCT- 614
AN1551.2 364 -GFD---GARSQFKDITNW---GQWDLVSLIVVG--NEVVTSNIA 399
AN1804.2 481 PRNGTFEA-NFHAGSLAFNA--TEKARQAKIYSSLP-TIVDIILD 521
A.xylinus 451 -GM--------DAPSMHLDA--NADALITAVAAANPRTVVVMETG 484
cons 901 945
AnBg11 533 GPVLVNEWYDNPNVTAILWGGLPGQESGN----------SL 563
AaBg11 532 GPVLVDEWYDHPNVTAILWAGLPGQESGN----------SL 562
Afu6g12010 182 NPIDVSPFVN--EVDAILNAHFPGQEGGQ----------AI 210
NFIA_027390 490 GPIILETILAQPSVKAIVWAGLPGQESGN----------AL 520
AFL2G_06408 514 GIRTVDAWIDHPNVTAVLFAGLPGQENGH----------SL 544
AO090701000841 514 GIRTVDAWIDHPNVTAVLFAGLPGQESGH----------SL 544
NFIA_098520 525 GV-NIMPWADNENVTAILAAHYPGQESGN----------SI 554
AO090005000337 522 GAVIMESWKD--RVPALLISWYSGSEGGH----------GL 550
AN2227.2 607 TPV-TMPWAP--KAKALLQAWFGGNECGN----------GI 634
AN2612.2 621 TPFNMLPWAD--NVKTHLHAWFGGNELGN----------GI 649
AN0712.2 615 GVAVAMPWLD--KVKAVVQAWFPGQEAGN----------AI 643
AN1551.2 400 SAAQLASFV-SEGASAFSAAGYTGQVTTAEPIDVWLSNGATLCPV 443
AN1804.2 522 RPAVIPEVVE--QAQAVLASYG--S-DSE----------AF 547
A.xylinus 485 DP-VLMPWNS--SVAGVLEAWFPGSGGGP----------AI 512
cons 946 990
AnBg11 564 ADVLYGRVNPGAKSPFTWGKTREAY--QD-YLYTE--P----NNG 599
AaBg11 563 ADVLYGRVNPGAKSPFTWGKTREAY--GD-YLVRE--L----NNG 598
Afu6g12010 211 ANILTGKTTPSGRLATTWPKKFDEEHVPT-YHNFPAR------LT 248
NFIA_027390 521 VDVIYGDTAPSGKLPYTIAKQAADY--GA-SW---------- 549
AFL2G_06408 545 VDILYGDVSPSGRLPFTVAKNESDY--GN-LLNST--V-----SF 579
AO090701000841 545 VDILYGDVSPSGRLPFTVAKNESDY--GN-LLNST--V-----SF 579
NFIA_098520 555 TDILWGNVNPSGKLPYTIARDSSDY--NTPILNLSGPA----AAR 593
AO090005000337 551 GDVLLGKVDASGRLPFSIPTSEAYL--P--FFNR---------- 580
AN2227.2 635 ADVLYGNVNPSGKLPLTFPVRLQDN--PS-YLNFR---------- 666
AN2612.2 650 ADVLFGVVNPSGKLPLSFPRRIEDT--PT-YLNFG---------- 681
AN0712.2 644 ADVLTGAVNPSGRLPVSFPRAIEDA--PA-HGNFPGDY-TDGKDN 684
AN1551.2 444 VDILGANLHPFFNPEFTAAEAGT---------------------- 466
AN1804.2 548 LDVVFGVSKPEGKLPFDLPRSMDAV--E--AQA---------- 576
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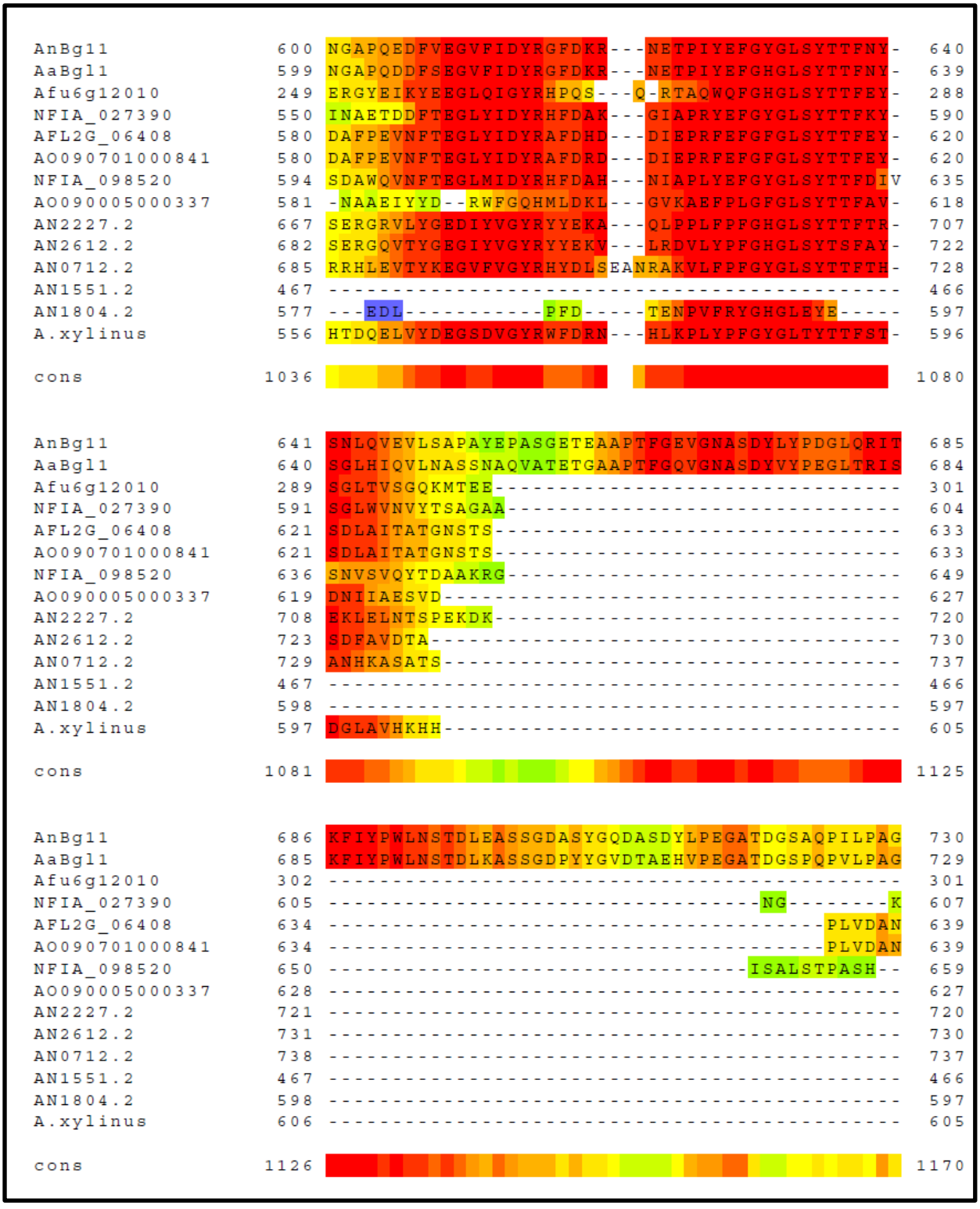
cons 991 1035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Afu6g12010 249 ERGYEIKYEEGLQIGYRHPQS---Q-RTAQWQFGHGLSYTTFEY- 288
NFIA_027390 550 INAETDDFTEGLYIDYRHFDAK---GIAPRYEFGYGLSYTTFKY- 590
AFL2G_06408 580 DAFPEVNFTEGLYIDYRAFDHD---DIEPRFEFGFGLSYTTFEY- 620
AO090701000841 580 DAFPEVNFTEGLYIDYRAFDRD---DIEPRFEFGFGLSYTTFEY- 620
NFIA_098520 594 SDAWQVNFTEGLMIDYRHFDAH---NIAPLYEFGYGLSYTTFDIV 635
AO090005000337 581 -NAAEIYYD--RWFGQHMLDKL---GVKAEFPLGFGLSYTTFAV- 618
AN2227.2 667 SERGRVLYGEDIYVGYRYYEKA---QLPPLFPFGHGLSYTTFTR- 707
AN2612.2 682 SERGQVTYGEGIYVGYRYYEKV---LRDVLYPFGHGLSYTSFAY- 722
AN0712.2 685 RRHLEVTYKEGVFVGYRHYDLSEANRAKVLFPFGYGLSYTTFTH- 728
AN1551.2 467 ---------- 466
AN1804.2 577 ---EDL----------PFD-----TENPVFRYGHGLEYE----- 597
A.xylinus 556 HTDQELVYDEGSDVGYRWFDRN---HLKPLYPFGYGLTYTTFST- 596
cons 1036 1080
AnBg11 641 SNLQVEVLSAPAYEPASGETEAAPTFGEVGNASDYLYPDGLQRIT 685
AaBg11 640 SGLHIQVLNASSNAQVATETGAAPTFGQVGNASDYVYPEGLTRIS 684
Afu6g12010 289 SGLTVSGQKMTEE---------- 301
NFIA_027390 591 SGLWVNVYTSAGAA---------- 604
AFL2G_06408 621 SDLAITATGNSTS---------- 633
AO090701000841 621 SDLAITATGNSTS---------- 633
NFIA_098520 636 SNVSVQYTDAAKRG---------- 649
AO090005000337 619 DNIIAESVD---------- 627
AN2227.2 708 EKLELNTSPEKDK---------- 720
AN2612.2 723 SDFAVDTA---------- 730
AN0712.2 729 ANHKASATS---------- 737
AN1551.2 467 ---------- 466
AN1804.2 598 ---------- 597
A.xylinus 597 DGLAVHKHH---------- 605
cons 1081 1125
AnBg11 686 KFIYPWLNSTDLEASSGDASYGQDASDYLPEGATDGSAQPILPAG 730
AaBg11 685 KFIYPWLNSTDLKASSGDPYYGVDTAEHVPEGATDGSPQPVLPAG 729
Afu6g12010 302 ---------- 301
NFIA_027390 605 ----------NG---------K 607
AFL2G_06408 634 ----------PLVDAN 639
AO090701000841 634 ----------PLVDAN 639
NFIA_098520 650 ----------ISALSTPASH-- 659
AO090005000337 628 ---------- 627
AN2227.2 721 ---------- 720
AN2612.2 731 ---------- 730
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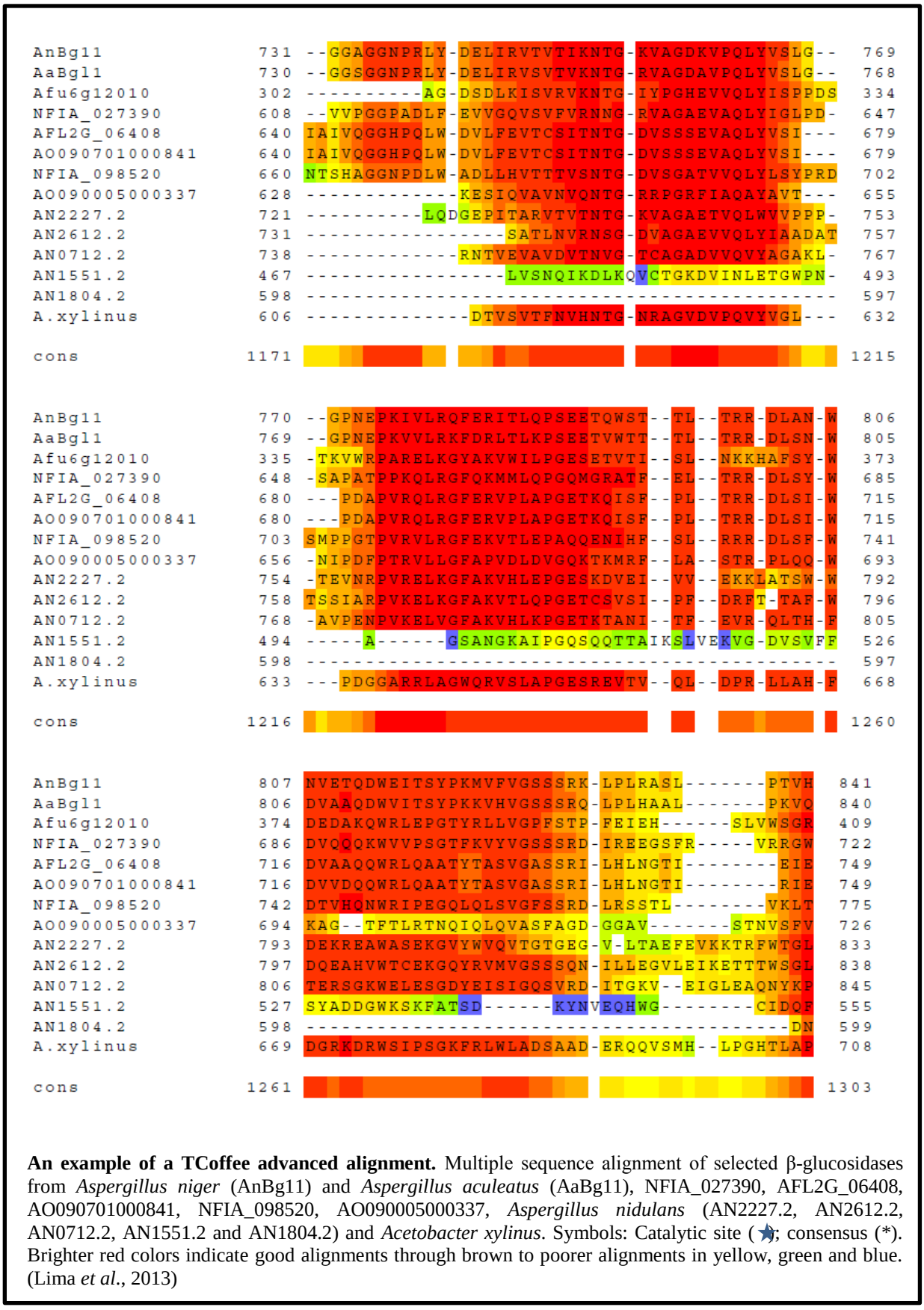
AN1804.2 598 ---------- 597
A.xylinus 606 ---------- 605
cons 1126 1170



An example of a TCoffee advanced alignment. Multiple sequence alignment of selected β-glucosidases from *Aspergillus niger* (AnBg11) and *Aspergillus aculeatus* (AaBg11), NFIA_027390, AFL2G_06408, AO090701000841, NFIA_098520, AO090005000337, *Aspergillus nidulans* (AN2227.2, AN2612.2, AN0712.2, AN1551.2 and AN1804.2) and *Acetobacter xylinus*. Symbols: Catalytic site (★); consensus (*). Brighter red colors indicate good alignments through brown to poorer alignments in yellow, green and blue. (Lima *et al.*, 2013)

Appendix 39: As seen in Kaduna. Source: Linda Ikeji blog, posted on October 25, 2015 at 11:13 am.

Appendix 40: Abstracts presented at conferences

a. Abstract presented at the Autumn SGM conference at University of Sussex, UK. 2nd – 4th September, 2013.

of by *E. coli* O157:H7 suggests the possibility of human exposure may be of a public health concern. Appropriate management of wastewater is necessary so that contamination of the environment and food by the organism can be prevented.

ENV/03

Mutating *E. coli* with the *arxA* gene: a novel, practical solution to the global arsenic water problem

Kriti Lall

Castilleja School, Palo Alto, CA, USA

Arsenic, a poison found in water, exists in the environment in mainly two states: arsenite (carcinogenic and water-soluble) and arsenate (easily removed from water), with arsenite being most predominant. Extremophilic bacteria such as MLHE-1 have a gene called *arxA*, which enables them to change toxic arsenite into less-toxic arsenate. In this study, a new bacteria strain was created by inserting *arxA* from MLHE-1 into a nonpathogenic, common bacteria, E. coli strain K-12. This mutated E. coli strain is an ideal choice for practical water bioremediation.

arxA was extracted from MLHE-1, and the gene was amplified using PCR. The gene was put on a plasmid using restriction digests and ligation; this plasmid was used to transform E. coli by heat shock. After confirming that the *arxA* gene was in the E. coli, the newly-mutated bacteria was tested for arsenite-to-arsenate conversion. Two samples of bacteria (normal E. coli and mutated E. coli) were subjected to 25 ppm arsenite media and analyzed over a set time interval. Arsenite and arsenate amounts in both media were compared in both samples at set time points. The mutated E. coli with the *arxA* gene (unlike the normal E. coli) successfully converted arsenite to arsenate.

ENV/04 ✓

Poly-gamma-glutamic acid (γ-PGA) – a promising biosorbent for removal of heavy metals

ADETORO OGUNLEYE, Craig Williams, David Hill, Isabella Radecka

University of Wolverhampton, Wolverhampton, UK

Poly-gamma-glutamic acid (γ-PGA), an unusual natural anionic biopolymer composed of D- and/or L-glutamic acid units polymerised through amide linkages between α-amino acid and γ-carboxylic acid groups, was synthesised by three bacterial strains – *Bacillus subtilis* (natto), *Bacillus licheniformis* 9945a and *Bacillus licheniformis* 9945. Three culture media – one containing glycerol, citric acid and L-glutamic acid as carbon sources, another, having citric acid, sucrose and L-glutamic acid as carbon sources and the third one with sucrose and L-glutamic acid as its sources of carbon were used in this study. Each strain produced γ-PGA extracellularly when grown aerobically in one or all three media. The biopolymers produced were identified as γ-PGA by Fourier transform infrared spectroscopy (FTIR). The effects of different fermentation temperatures (37°C and 50°C) and media on bacterial growth, production and molecular weight of γ-PGA were investigated. The metal binding affinity of γ-PGA was also studied and it was found that it binds heavy metals. The optimal γ-PGA yield of 11.45g/l was obtained when *Bacillus subtilis* (natto) was grown aerobically at 37°C for 96 hours in a culture medium containing having citric acid, sucrose and L-glutamic acid as carbon sources.

ENV/05 ✓

Bioremediation of crude oil-contaminated soil using bacteria and zeolite

WILLIAMS JOSEPH, David Hill, Iza Radecka, Clive Roberts

University of Wolverhampton, Wolverhampton, UK

Bioremediation is an important, cost effective and environmental friendly method used to clean up the soil and the environment from petroleum hydrocarbon contaminants utilising indigenous or selected microbial flora. The bioremediation of crude oil artificially contaminated soil by a mixed culture of two hydrocarbon-degrading bacteria, Rhodococcus spp and Pseudomonas spp, was investigated. These bacterial strains were selected based on criteria that they were able to utilise hydrocarbons as the sole source of carbon and energy and were able to show significant growth in crude oil. The influences of a zeolite (clinoptilolite) and inorganic nutrient additions on the biodegradation of crude oil in soils were investigated. Soil amendment experiments at 30°C for a period of 30 days showed a more rapid and greater extent of apparent oil removal with the addition of both bacterial consortium and clinoptilolite. There was 79% oil removal by the bacterial consortium in the soil amended with clinoptilolite as compared to 67% in the case of the amended soils without clinoptilolite. Although, the addition of both bacterial consortium and clinoptilolite enhanced the removal of the crude oil, however the effect of clinoptilolite may be one of abiotic removal.

FB
Fermentation and bioprocessing (industry) ✓

FB/01

Purification and characterisation of novel recombinant β-glucosidases from *Aspergillus*

RICHARD AUTA, Paul Hooley, Iza Radecka

University of Wolverhampton, Wolverhampton, West Midland, UK

An initial bioinformatics analysis of the genome databases for β–glucosidases for *Aspergillus* has collated the variation in this enzyme class using keyword searches and Blast. Selection of novel candidates for direct gene synthesis for cloning and expression is based on size (short genes), pI (ExPASy – Tools) and novelty. Several *Aspergillus* strains have been screened using a rapid plate assay based on Congo Red. Selection of candidate strain was based on temperature profile, pH range and carbon source degradation. Potential bacterial donors of cellulose degrading enzymes have also been explored for their expression in fungal hosts. *Pichia pastoris* systems will be used for enhanced expression of *Aspergillus* proteins employing fusion PCR of the target gene with an inducible promoter. At the end of this work we hope to generate new β-glucosidases, cloned and analyzed for industrial use to produce biofuel (renewable energies).

FB/02

Investigation of the control of antibiotic production and sporulation in *Streptomyces coelicolor*: the role of specific small non-coding RNAs

OUSAMA ALSHANAA

The University of Surrey, Guildford, UK

RNA-binding chaperone proteins in *Streptomyces coelicolor* A3 (2) MT1110 are investigated in this experiment. The aim is to discover chaperone proteins involved the post-transcriptional regulation of gene expression in the biotechnologically vital streptomycetes bacteria. Depending on experiments confirming the expression of many small non-coding RNAs in *S. coelicolor* A3 (2), scr3558-1 RNA was chosen for this experiment to find what chaperone proteins bind to these RNAs *in vitro*.

scr3558-1 gene was identified and cloned in pUC18 plasmid. T7 promoter was cloned upstream to *scr3558* and tobramycin aptamer downstream of the gene. The clone was PCR amplified and *in vitro* transcribed. scr3558-1 RNA was ligated to

b. Abstract presented at the 16th European congress on Biotechnology. 13th – 16th July, 2014 at EICC Edinburgh, Scotland.

Glycobiology

PJ-01

Cloning and characterization of novel β-glucosidases from *Aspergillus* and their functional expression in methylotrophic yeast *Pichia pastoris*

Richard Auta*, Paul Hooley, Iza Radecka

University of Wolverhampton

The production of fermentable sugars from lignocellulosic material has attracted interest in the optimization of conditions related to conversion of cellulose to glucose for biofuel production. *Aspergillus* strains are known as efficient producers of β-glucosidase which is a rate limiting factor during enzymatic hydrolysis of cellulose.A bioinformatics based approach to characterize β-glucosidase encoding enzymes in the genus *Aspergillus* is described and the application of bioinformatics in the selection and expression of target genes is explained. Five *Pichia* clones (carrying *A. nidulans* AN2227.2, AN2612.2, AN0712.2, AN1551.2 and AN1804.2 in pPICZ vectors) that exhibit satisfactory levels of expression of recombinant β-glucosidase were obtained from the Fungal Genetics Stock Centre (FGSC). A study to compare their hydrolytic activities and relate these to their growth profiles using different media was carried out. The characteristics of these enzymes and the use of *P pastoris* in the expression of these proteins are discussed

Keywords

β-glucosidase; Aspergillus; Bioinformatics; Hydrolysis

http://dx.doi.org/10.1016/j.nbt.2014.05.2006

PJ-02

Kinetic evaluation of carbohydrate-protein interaction using SPR

Jeong Hyun Seo[1,*], Chang Sup Kim[2], Hyung Joon Cha[2]

[1] Yeungnam university
[2] POSTECH, Korea

Surface plasmon resonance (SPR) can provide kinetic information about an interaction, and it can also be used to rapidly monitor dynamic processes, such as adsorption and degradation, without the need for sample labeling. Here, we employed SPR to analyze carbohydrate-protein interactions, particularly GM1-related carbohydrate-*Vibrio cholera* toxin interactions. The interaction between cholera toxin subunits A (ctxA) and B (ctxB) was similar to general ligand-receptor interactions. After the direct immobilization of thiol-containing GM1 pentasaccharide on a gold surface, the GM1-ctxB interaction kinetics were evaluated, and they showed a similar degree of kinetics as reported in previous reports. We found that ctxA had a high affinity for the GM1-ctxAB complex, although its equilibrium dissociation constant was 10-times lower than that of GM1-ctxB binding. Comparative analyses for GM1-related carbohydrates-ctxAB interactions were also conducted to determine the kinetic values of several GM1 analogues with different structures, although their kinetic values were one-order of magnitude lower than those of the GM1-ctxAB interaction. The kinetic analysis results for the interactions of GM1 analogues and ctxAB indicated that the sialic acid thumb is important for recognition, and the terminal galactose and N-acetylgalactosamine finger are required to stabilize the GM1-ctxAB interaction. Taken together, our results indicate that the direct immobilization of carbohydrate in an SPR-based analytical system can be used to evaluate the structural contribution of carbohydrate moieties in carbohydrate-protein interactions, as well as provide valuable information that can be used to understand the interactions.

http://dx.doi.org/10.1016/j.nbt.2014.05.2007

c. **Abstract presented at the International Union of Microbiological Societies Congresses. July 27 – August 1, 2014 at Montreal, Canada.**

Tuesday, 29 July 2014 11:00 - 12:00 Room 516/517 A-B

Poster Session

MEM-PT3001 - A bioinformatics – based characterization of β-glucosidases (GH1 and GH3) in the genus Aspergillus

Richard Auta[1], Paul Hooley[1], Iza Radecka[1]
[1]*University of Wolverhampton, Wolverhampton, UK*

The conversion of cellulose to fermentable sugars is highly dependent on the use a range of efficient enzymes. β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose. A bioinformatics based approach to characterize β-glucosidase (GH1 and GH3) encoding enzymes in the genus Aspergillus is described. Each species encodes between 7 and 27 β-glucosidases. Multiple sequence alignments and Phylogenetic tree analysis highlight examples of candidate encoding genes horizontally transferred from bacteria. The application of bioinformatics in the selection and expression of target genes is explained.

A bioinformatics – based characterization of β-glucosidases (GH1 and GH3) in the genus *Aspergillus*

R. Auta, P. Hooley, I. Radecka E-mail: r.auta2@wlv.ac.uk
Faculty of Science and Engineering, University of Wolverhampton, UK

Introduction

The production of fermentable sugars from lignocellulosic material has attracted interest in the optimization of conditions related to conversion of cellulose to glucose for biofuel production (Fig. 1). Alternative energy technology is important because of the rising prices of crude oil and environmental issues such as global warming and air pollution.

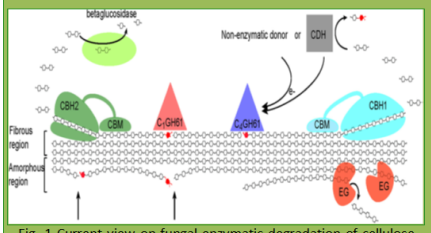


Fig. 1 Current view on fungal enzymatic degradation of cellulose. Abbreviations: EG, endoglucanase; CBH, cellobiohydrolase; CDH, cellobiose-dehydrogenase; CBM, carbohydrate-binding module (Horn *et al.*, 2012) .

Why β-glucosidase?

β-glucosidase is responsible for the regulation of the whole cellulolytic process and is a rate limiting factor during hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose accumulation (Harhangi *et al.*, 2002).

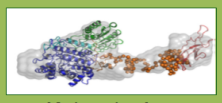


Fig. 2: Structure of β-glucosidase from *Aspergillus niger* (AnBgl1), Lima *et al*, 2013.

A paper by Lima *et al.*, 2013 revealed that β-glucosidase from AnBgl1 has a tadpole-like structure (Fig. 2).

Aims

This research focuses on the use of bioinformatics tools to identify β-glucosidases with novel properties.

Methods

Computer aided bioinformatics was used to provide a rapid means for identifying β-glucosidase with novel characteristics.

Acknowledgment: This research work is sponsored by PTDF.

Results and Discussion

Species	Gene Name		Family						Gene product	
	Hypothetical β-glucosidase	Known β-glucosidase	GH1	GH3	GH5	GH17	GH31	No *	Named	Unnamed
A. flavus	21	1	2	14	1	2	-	8	22	-
A. nidulans	8	24	2	12	1	1	2	7	27	-
A. fumigatus	11	14	5	12	1	2	1	4	-	35
A. terreus	15	2	2	9	1	1	-	4	17	-
N. fischeri	23	-	9	12	1	1	1	8	28	-
A. clavatus	18	1	3	7	1	2	2	3	17	-
A. oryzae	20	-	2	10	1	2	2	5	20	-
A. niger	7	-	1	2	1	-	-	3	-	7

Table 1: Summary of manual search from 8 species from *Aspergillus* comparative database (http://www.broadinstitute.org). GH: Glycoside Hydrolase; GH1 & GH3: β-glucosidases; No*: family name not indicated. Each sequence was manually checked for active sites (Lima *et al*, 2013)

CAZy classification of GH has placed β-glucosidase in GH family 1 and 3 based on their amino acid sequence structure. GH5, GH17 and GH31 could be accessory enzymes that are co-expressed with β-glucosidase for efficient hydrolysis of cellulose during growth on cellulosic substrates. The non-classification of some β-glucosidases could be as a result of annotation errors.

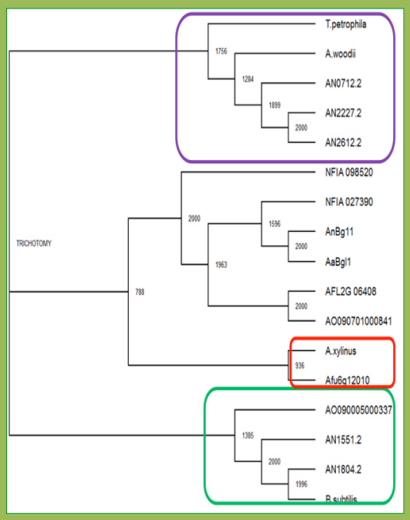


Fig. 3: Treeview analysis of fungal enzymes from *A. niger* (AnBg11) and *A. aculeatus* (AaBg11), NFIA_027390, AFL2G_06408, AO090701000841, NFIA_098520, AO090005000337, *A. nidulans* (AN2227.2, AN2612.2, AN0712.2, AN1551.2 and AN1804.2), and bacterial enzymes from *Acetobacter xylinus, Acetobacterium woodii, Thermotoga petrophila* and *Bacillus subtilis* (shown with values out of 2000 bootstraps for branch points).

Multiple Sequence Alignment (MSA) and phylogenetic trees show an unexpected association between β-glucosidase gene products from *Aspergillus* and bacteria suggesting possible Horizontal Gene Transfer (HGT) events from bacteria to *Aspergillus.*

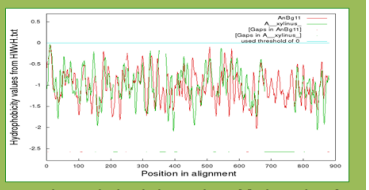


Fig. 4: AlignMe hydrophobicity plots of β-glucosidase from *A. niger* (AnBg11) and *Acetobacter xylinus.*

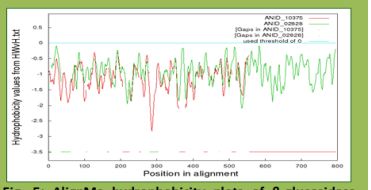


Fig. 5: AlignMe hydrophobicity plots of β-glucosidase GH1and GH3 family from *A. nidulans* (ANID_10375) and *A. nidulans* (ANID_02828) respectively.

Hydrophobicity plots (Fig. 4 and 5) were conducted to determine the hydrophobicity of the proteins. A negative number is hydrophilic while a positive number is hydrophobic. The results show similarity through all the proteins and revealed the proteins to be largely hydrophilic. The hydrophilic nature of β-glucosidase offer some unique advantages such as ease of dispersal.

Conclusions

- Bioinformatics studies have identified several new genes and for the first time compiled a complete list of β-glucosidases in the *Aspergillus* genus.
- The *Aspergillus* genus harbours unidentified β-glucosidase genes with attractive properties for cellulose degradation systems, some of which may be bacterial in origin.

Next action plan

- Genomic DNA cloning/synthesized gene expression in *Pichia pastoris.*
- Biochemical characterization of recombinant β-glucosidases.

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Poster presented at the International Union of Microbiological Societies Congresses. July 27 – August 1, 2014 at Montreal, Canada.

d. Abstract presented at the Experimental Biology Conference, March 26 – April 1, 2015 at Boston, USA

The FASEB Journal
The Journal of the Federation of American Societies for Experimental Biology

Production of Bacterial Cellulose, Cloning and Characterization of Novel β-glucosidases from *Gluconacetobacter xylinum*

Richard Auta[1,2], Joshua Loh[1], Iza Radecka[1] and Paul Hooley[1]

Author Affiliations

Abstract

Bacterial cellulose (BC) is produced by bacteria has a unique structural and mechanical properties and is highly pure as compared to plant cellulose. In this study, BC was produced using Hestrin–Schramm (HS) media. Two forms of cellulose were observed; a gel–like membrane and a leathery membrane. Fourier Transform infrared spectroscopy (FTIR) investigation revealed both forms to be cellulose and indicated the presence of CH_2 and OH group at the absorption wavelength of 3300 cm^{-1}, 1650 cm^{-1} and 1025 cm^{-1}. Scanning Electron Microscopy (SEM) results showed fine morphology of the cellulose matrix and fibre; and XRD indicated the majority of the cellulose to be type–1 cellulose (crystalline in nature). Plasmid DNA sequence analysis revealed an open reading frame of 678 bp. The significance of this work is discussed.

Production of Bacterial cellulose, cloning and characterization of novel β-glucosidases from *Gluconacetobacter xylinum*

*Richard Auta [a, b], Joshua Loh [a], Iza Radecka [a] and Paul Hooley [a]

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Introduction

Bacterial cellulose (BC – Figure 1A and B) is produced by bacteria such as *Gluconacetobacter xylinum* (*G. xylinum*). It has unique structural and mechanical properties and is highly pure as compared to plant cellulose. The properties make BC advantageous for biotechnology applications, for example in the production of biofuels. Intriguingly, *G. xylinum* strains which are known to be efficient producers of bacterial cellulose also produce β-glucosidase. β-glucosidase is a rate limiting factor during enzymatic hydrolysis of cellulose; this is due to the fact that endoglucanase and exoglucanase which play an important role during cellulose hydrolysis are often inhibited by accumulation of cellobiose (Harhangi *et al.*, 2002).
The objective of this work is to clone and characterize a novel β-glucosidase from *G. xylinum* and to produce BC for biofuel production.

Fig. 1A: Wet Bacteria cellulose (BC) pellicle being removed from a culture; 1B: Washed Bacterial cellulose (BC).

Materials and Methods

Culture medium and growth conditions

Schramm and Hestrin (1954) medium (HS medium) was used to produce BC from *G. xylinum* under static conditions.

FTIR, SEM and XRD studies

The cellulose obtained from HS medium was analysed by FTIR spectrometer. The cellulose fibril was characterized using the Scanning Electron Microscope (SEM). XRD analysis was utilised to characterise the crystallinity index and the degree of polymerisation of the BC sample.

DNA isolation and PCR conditions

G. xylinum genomic DNA was extracted using PureLink™ Genomic DNA Mini kit (Catalogue No. K1820-00). The extracted genomic DNA was used as a template for PCR reactions.

Cloning and sequencing of the *G. xylinum* β-glucosidase gene

The amplified gel extracted PCR products were purified and ligated into pGEM-T Easy vector systems (Promega) using DNA ligase and ligation buffer by the method of TA cloning following the manufacturer's instructions. Sample pDNA was sequenced for inserts at Source BioScience LifeSciences (Rochdale, United Kingdom).

Appreciation

Authors thank Petroleum Technology Development Fund (PTDF) for their financial support

Results and discussion

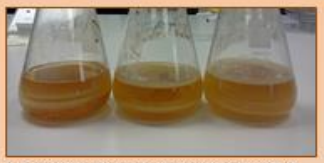

Fig. 2: Layers of bacterial cellulose formed by *G. xylinum* in triplicate

The BC samples used for analysis were harvested from a single flask containing multiple layers of BC (Figure 2).

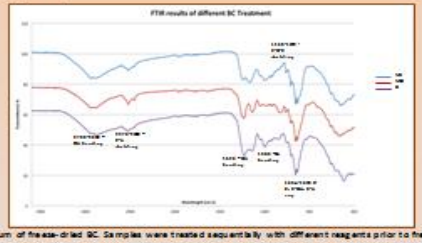

Fig. 3: FTIR spectrum of freeze-dried BC. Samples were treated sequentially with different reagents prior to freeze drying. NSD: Sample treated with NaOH, SDS and washed with dH_2O, ND: Sample treated with NaOH and washed with dH_2O, D: Sample washed with dH_2O only

The FTIR results (Fig. 3) indicated that all samples contained functional groups that were associated with cellulose, such as: 3700 - 3000 cm^{-1} hydrogen bonding -OH bending, 2970 - 2800 cm^{-1} -CH symmetrical stretching and 1034 - 1023 cm^{-1} C-C, C-OH and C-H ring side group vibrations, which is typically widely reported for bacterial cellulose (Fan *et al.*, 2012).

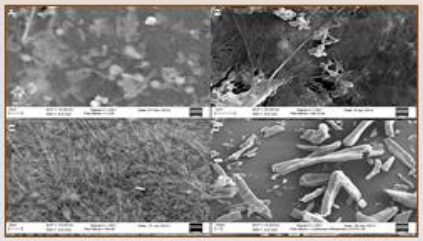

Fig. 4: SEM collection of BC micrograph. A: Rod-shaped cells of *G. xylinum* (approx 1.5-2µm in length) entangled in BC, B and C: NaOH treated and washed cellulosic fibrils at different magnification, D: The non-microbial cellulose sample from Whatman.

SEM (Fig. 4) revealed a complex mesh of cellulose ribbons that interweaved among each other to form the BC membrane.

Fig. 4A is an SEM image of BC showing distinct rod-shaped cells entangled by the fine cellulose ribbons. The length of each cell is roughly 1 – 2 µm. The dendritic nodes which were widespread throughout the sample (Fig. 4 B and C) in a variety of shape and sizes were amorphous in nature indicating the presence of type II cellulose (Sarkar and Perez, 2012).

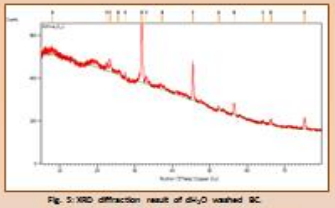

Fig. 5: XRD diffraction result of dH_2O washed BC.

BC diffractogram (Fig. 5) reveals two principal diffraction peaks at 15° and 29° confirming the presence of type-1 cellulose (Czaja *et al.*, 2004; Moosavi-Nasab and Yousefi, 2011; Sheykhnazari *et al.*, 2011).

Fig. 6: Plasmid DNA of AP012159.1 (A - High ranger ladder, B - Control, C, D and E – AP012159.1 pDNA (10^{-4}, 10^{-5}, 10^{-6} – pDNA dilutions), F - PCR product ranger ladder)

Plasmid DNA amplicons obtained were sharp (Fig.6) and BLAST analysis gave 80% identity and 49% coverage to *G. xylinus* 3288 (expectation value of 3 e-60) . Manual analysis of the nucleotide sequence open reading frame indicated the sequence to be truncated by several stop codons suggesting that the gene may be non functional.

Conclusion

In this study, BC was produced using HS media. FTIR investigation indicated the presence of CH_2 and OH group while SEM results showed fine morphology of the cellulose matrix and fibre. XRD indicated the majority of the cellulose to be type-1. The β-glucosidase gene of *G. xylinum* was successfully cloned and its nucleotide sequence of 678 bp was determined. Further study is required for characterization of the recombinant enzyme from *G. xylinum* after its expression.

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e. Abstract presented at the Society for General Microbiology Annual Conference. March 29 – April 2, 2015 at Birmingham International Conference Centre (ICC), United Kingdom.

S08/05

Cloning of novel β-glucosidases gene from *Gluconacetobacter xylinum*

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The following study presents the production of bacterial cellulose (BC) and cloning of β-glucosidase gene from Gluconacetobacter xylinum (G. xylinum) strain. In this study, BC was produced using Hestrin-Schramm (HS) media. Fourier Transform infrared spectroscopy (FTIR) investigation indicated the presence of -CH and -OH group at the absorption wavelength of 3300 cm^{-1}, 1620 cm^{-1} and 1025 cm^{-1}. Scanning Electron Microscopy (SEM) results under various magnifications showed fine morphology of the cellulose matrix and fibre; and XRD indicated the majority of the cellulose to be type-1 cellulose (crystalline in nature). Intriguingly, G. xylinum strains which are known to be efficient producers of bacteria cellulose also produce β-glucosidases. The encoding gene is located in the cellulose synthase operon. The genomic DNA of G. xylinum was isolated and employed for the PCR amplification. PCR primers were designed to flank the β-glucosidase gene of G. xylinum and PCR products of the correct length were cloned in E. coli using pGEM-T. Plasmid DNA sequence analysis revealed an open reading frame of 678 bp. BLAST analysis confirmed as the nearest significant match (expectation value of 3 e^{-60}) the G. xylinum 3288 β-glucosidase (AP012159).

Keywords: β-glucosidase gene, PCR amplification, Cloning, DNA sequencing, G. xylinum

S08/07

Molecular mechanisms of HSV-1 egress: the roles of viral proteins UL7 and UL51

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Efficient envelopment and egress of herpes simplex virus 1 (HSV-1) from infected cells requires the viral tegument proteins UL7 and UL51, both of which are conserved across herpesvirus subfamilies. Deletion of either UL7 or UL51 in HSV-1 results in a small plaque phenotype, which is also observed upon deletion of homologous proteins in pseudorabies virus (PrV) and human cytomegalovirus (CMV). Unpublished work form our labs has identified an interaction between UL7 and UL51 by yeast-two-hybrid screen (Y2H). Another study has shown UL51, a membrane-associated protein, to recruit UL7 to cytoplasmic membranes and UL51 is also required for the efficient incorporation of UL7 into mature virions. Using recombinant proteins we have shown that the U44 domain of UL51 mediates a direct interaction with UL7 in vitro and that this interaction is conserved in at least one other herpesvirus, murid herpesvirus. Protein expression screens for UL7, UL51 and their homologues have yielded several soluble proteins amenable for structural and biophysical interaction studies. Progress toward a structural and functional understanding of the roles of UL51, UL7 and their homologues in herpesvirus maturation will be discussed.

S08/10

Roles of the vaccinia virus proteins F12 and E2 in viral egress

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Vaccinia virus (VACV) utilises microtubule-mediated transport at three stages of its replication cycle. Of these the transport of fully formed intracellular enveloped virions (IEVs) during virion egress to the cell surface is the best-characterized process, although the regulatory mechanisms and viral and host proteins involved are not well understood. VACV proteins F12 and E2 function as a complex and are both required for IEV egress. Here we report that the F12/E2 complex interacts with the kinesin-1 motor protein complex through a specific interaction with kinesin light chain (KLC) isoform 2. E2 is necessary and sufficient for this interaction. To characterize this interaction further, we constructed KLC1-KLC2 chimeras and determined the ability of these to bind to the F12/E2 complex. This showed that the specificity determining region for F12/E2 was the variable C-terminal tail (CT). However, siRNA knockdown of KLC2 did not prevent virion egress, while KLC1 knock-down increased virion egress efficiency. Our study shows a role for the F12/E2 complex in kinesin-1 mediated IEV egress, although their exact roles remain unclear. We hypothesise that the F12/E2 complex is able to target the complete IEV for egress via interacting with kinesin-1 complexes containing specific subset of KLC isoforms.

S09/01

Metabolic capacity of mitochondrion-derived organelles in the free-living anaerobic stramenopile *Cantina marsupialis*

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Functionally and morphologically degenerated mitochondria, so-called mitochondrion-derived organelles (MDOs), are frequently found in eukaryotes inhabiting in oxygen-depleted environments. MDOs have been discovered from a

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M006

ACETYLCHOLINESTERASE ACTIVITY IN THE BRAIN AND BLOOD OF MICE INFECTED WITH *Plasmodium berghei berghei*

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Acetylcholinesterase (AChE) has been implicated in cerebral malaria, which thus, is the cause of 12% of psychiatry disorder and a leading cause of death in sub-Saharan Africa. This study was designed to investigate AChE activity in the blood and brain of mice infected with plasmodium *berghei berghei (P.b. berghei)*. Twenty five (25) mice were divided into 5 groups (A - E) of 5 mice each. Group A - D were administered with *P.b.berghei* for 4 days, 8 days, 12 days and 5 days/ treated with 100mg/kg/day artemisinin. Group E serve as the negative the control (not infested and not treated). The parasiteamia level, PCV and AChE activity was determined using acetythiocholine (ACTC) as substrate. There was significant ($p^{<}$ 0.05) decrease in AChE activity in the brain (1.19×10^{-3} µmolACTCmin^{-1}mgprotein^{-1}) and blood (1.47×10^{-3} µmolACTCmin^{-1}mgprotein^{-1}) of mice infected for 12 days, as compared with the control (6.88 and 6.62×10^{-3} µmolACTCmin^{-1}mgprotein^{-1} respectively). The activity of AChE in the brain and blood (4.47×10^{-3} µmolACTCmin^{-1}mgprotein^{-1} and 4.28×10^{-3} µmolACTCmin^{-1}mgprotein^{-1} respectively) of mice infected and treated *in-vivo* for 5 days with 100mg/kg/day artemisinin were significantly reduced as compared with the control. There was significant ($p^{<}$ 0.05) reduction in the PCV of the entire infested groups as compared with the control group (53.34 %), however, artemisinin treated group producesa promising 45.73 % PCV. Parasitemia level in group A-C increases with days, but reduces in group D. This finding reveals that both the brain and blood AChE activity decreases during infection of *P.b. berghei*, but however, was reversed upon administration of artemisinin. Thus, suggest the promising potentials of Artemisinin in mitigating the complications associated with malaria.

Keywords: *Plasmodium b.berghei, Acetylcholinesterase, cerebral malaria, parasitemia*

M007

PURIFICATION AND CHARACTERIZATION OF A B-GLUCOSIDASE FROM *Aspergillus nidulans* AN1804 EXPRESSED IN*Pichia pastoris*

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This study reports the purification and characterization of recombinant β-glucosidase from *Pichia pastoris* clones carrying an *Aspergillus nidulans* (*A. nidulans*) AN1804 gene that exhibits satisfactory level of expression of the protein. The clone was obtained from Fungal Genetic Stock Centre USA. Purification was conducted using ammonium sulphate precipitation and anion exchange chromatography on a DEAE-Sephadex A-50 column. The enzyme was purified 2.25 fold from the crude extract with a 33.33% recovery yield. The protein migrated homogeneously as a single band on SDS-PAGE stained with Sterling rapid silver stain and the molecular weight was determined to be approximately 100 kDa. It was optimally active at 50 °C and pH 5.5, though it had a broad pH range of pH 3.0 – 10.0. The presence of $CoCl_2$, $CaCl_2$, $FeCl_3$, $FeCl_2$ and $ZnCl_2$ slightly enhanced the activity of β-glucosidase but was slightly decreased in the presence of $HgCl_2$. β-glucosidase showed very high affinity to para-Nitrophenyl β-D-glucopyranoside (pNPG). K_m and V_{max} for the hydrolysis of pNPG by β-glucosidase was calculated as 0.59 mM and 2.04 µmole/ml/min respectively.

Keywords: β-glucosidase, *A. nidulans*, *P. pastoris*, para-Nitrophenyl β-D-glucopyranoside

g. **Abstract presented at the 10th Federation of African Societies of Biochemistry and Molecular Biology (FASBMB) Congress/34th Nigerian Societies of Biochemistry and Molecular Biology (NSBMB) Conference on the November 2nd – 6th, 2015 at Minna, Nigeria.**

M008

PRODUCTION AND CHARACTERIZATION OF BACTERIAL CELLULOSE (BC) BEFORE AND AFTER ENZYMATIC HYDROLYSIS

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Bacterial cellulose (BC) is produced by bacteria such as *Gluconacetobacter xylinum* (*G. xylinum* 639). It has unique structural and mechanical properties and is highly pure as compared to plant cellulose. These properties make BC advantageous for biotechnology applications. This study aimed at the production of BC from *G. xylinum* 639 strains and attempt the degradation of the BC produced using commercial fungal cellulase enzyme. BC was produced in Hestrin-Schramm (HS) medium. *G. xylinum* produced an average dry yield of 1.4 ± 0.09 g/L BC after 9 days of fermentation. Scanning Electron Microscopy (SEM) analysis confirmed BC to be highly structured while Fourier Transform infrared spectroscopy (FTIR) analysis indicated that the absorption peaks at 3000 – 3700, 2800 – 2970 and 1023 – 1024 cm^{-1} were derived from –OH bending, -CH stretching and C-C/C-OH/C-H ring vibrations: X-Ray Diffraction (XRD) revealed a high purity of BC indicating type I cellulose with a high crystalline nature. The solid state ^{13}C NMR spectroscopy of the untreated BC sample also indicated high crystallinity while the cellulase treated BC sample was dominated by a polysaccharide signal between 55 and 110 ppm. Differential Scanning Calorimeter (DSC) analysis of an untreated BC sample was stable up to 200 °C, above which decomposition started with a pronounced disintegration at 338.13 °C. Thermogravimetric analysis (TGA) showed that sample moisture content was present only at temperatures below 100 °C and the weight loss of hydrolysed BC was faster than the untreated BC. The significance of this study for recycling biotechnology developments is discussed.

Keywords: Bacterial cellulose, SEM, FTIR, XRD, TGA, DSC, cellulase enzyme

M009

TRANSPORT BIOENERGETICS OF AMINO ACIDS ACROSS VITELLINE MEMBRANE INTO YOLK OF *Gallus domesticus*

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Amino acid (AA) compositions of raw egg white and yolk of *Gallus domesticus* were determined and their free energy of transport (ΔG_t) across vitelline membrane into yolk calculated. Results indicate that transport of Na^+ in the egg white into yolk can release a ΔG_t of −10.80 ± 0.06 kJ/mol. Transport of Arg, Thr, Ser, Gly Ile and Ala into yolk can proceed spontaneously by facilitated diffusion down their concentration gradients, while transport of Lys, His, Asp, Asn, Glu, Gln, Pro, Cys, Val, Met, Leu, Tyr and Phe into yolk will require input of ΔG_t. Charged AAs (at pH ~7) establish equilibrium potentials while their non-charged counterparts cannot. Transporting the Arg group of AAs can release a total ΔG_t ($\sum \Delta G_t$) of −7.02 kJ/mol, whereas transporting the Lys group of AAs will require a $\sum \Delta G_t$ of + 25.56 kJ/mol. The Lys group of AAs will each require ΔG_t got by cotransport with one mole of Na^+ in the egg white as it moves, down its concentration gradient, into yolk. In conclusion, the free energy released when three moles of Na^+ in the egg white are transported into yolk can sufficiently transport all the active transport-requiring amino acids into the yolk.